


GLUCOSE TRANSPORTER-4 ON PERIPHERAL BLOOD MONONUCLEAR CELLS IN  
CONDITIONED VS. SEDENTARY COLLEGE STUDENTS

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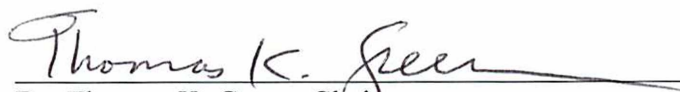
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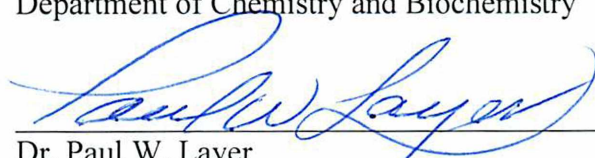
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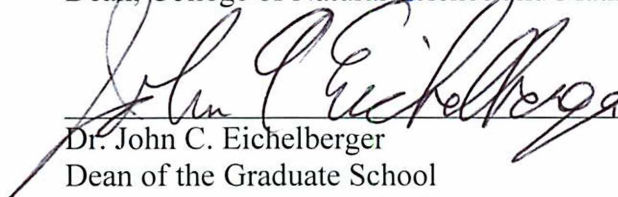
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GLUCOSE TRANSPORTER-4 ON PERIPHERAL BLOOD MONONUCLEAR CELLS IN  
CONDITIONED VS. SEDENTARY COLLEGE STUDENTS

A DISSERTATION

Presented to the Faculty  
of the University of Alaska Fairbanks

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DOCTOR OF PHILOSOPHY

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## Abstract

Glucose transporter 4 (GLUT-4) plays a key role in the pathophysiology of type 2 diabetes. GLUT-4 is upregulated in response to exercise, enhancing cellular glucose transport in skeletal muscle tissue. This mechanism appears to remain intact in individuals with insulin resistance. There is evidence of increased translocation of GLUT-4 and increased transcription of *SLC2A*, the gene which codes for GLUT-4. Details of the mechanism are poorly understood and are challenging to study due to the invasive nature of muscle biopsy. Peripheral blood mononuclear cells (PBMC) have documented insulin-sensitive GLUT-4 activity and may serve as a proxy tissue for studying skeletal muscle GLUT-4. The purpose of this study was to investigate whether GLUT-4 on PBMC is affected by exercise in a similar fashion to myocytes. Additionally, correlations between PBMC GLUT-4 and common indicators of insulin resistance and dietary patterns were examined. The results show a trend toward higher PBMC GLUT-4 levels in conditioned athletes than in their sedentary counterparts, similar to what has been documented in myocytes. Females were shown to have higher PBMC GLUT-4 levels than males. *SLC2A4* mRNA analysis demonstrates a difference in mean gene expression between the conditioned and sedentary participants. Correlations between levels of PBMC GLUT-4 and hemoglobin A1c (HbA1c), glucose, insulin, HOMA-IR, BMI, or body fat were not detected. Relationships between specific nutrients and GLUT-4 were also not detected. This study provides evidence to support exploration of PBMC as a proxy tissue for studying GLUT-4 response to exercise or other non-insulin factors. This could provide important treatment avenues for individuals with insulin resistance and type 2 diabetes.



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AA	Arachidonic acid
AICAR	5-Aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside
AMPK	5' AMP-activated protein kinase
AMPM	Automated multiple pass method
BMI	Body mass index
CaMK	Calmodulin-dependent protein kinase
CLA	Conjugated linoleic acid
C <sub>T</sub>	Cycle threshold
DHA	Docosahexaenoic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEF	GLUT-4 enhancer factor
GLUT-4	Glucose transporter-4
HAT	Histone acetyl transferase
HbA1c	Hemoglobin A1c (glycated hemoglobin)
HDAC	Histone deacetylase
HIF1 $\alpha$	Hypoxia induced factor 1 $\alpha$
HOMA-IR	Homeostatic model assessment of insulin resistance
IPAQ-SF	International Physical Activity Questionnaire – Short Form
IR	Insulin resistance
IRS	Insulin receptor substrate proteins

IUGR	Intrauterine growth retardation
MEF	Myocyte enhancer factor
MET	Metabolic equivalent of task
NHANES	National Health and Nutrition Examination Survey
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
RT PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
<i>SLC2A4</i>	Solute carrier family 2 (facilitated glucose transporter), member 4
TRE	Thyroid response element
VO <sub>2</sub> max	Maximal oxygen consumption

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## Chapter 1: Glucose Transporter 4 (GLUT-4) and Glucose Metabolism Disorders

### 1.1 Type 2 Diabetes and Insulin Resistance

Diabetes is a pervasive health problem in the United States affecting an estimated 29.1 million people, a number which has tripled in the past three decades.<sup>1</sup> One in three people will develop diabetes in their lifetime. The disease has estimated direct and indirect costs of a staggering \$245 billion annually.<sup>1</sup> Of those diagnosed, over 95% have Type 2 diabetes. The vast majority of these individuals with Type 2 diabetes are on antidiabetic medications.<sup>1</sup> Unfortunately, 8.1 million people who have the disease do not even know they have it.<sup>1</sup>

Central to the pathophysiology of diabetes is insulin resistance whereby the cells, particularly muscle and adipose cells, do not utilize insulin efficiently. This leads to elevated blood glucose levels, beginning the pathology of diabetes. Prediabetes is the condition where blood glucose is elevated above normal levels, but not yet to the threshold for the diagnosis of diabetes. Of the 86 million Americans with prediabetes, nine out of ten are undiagnosed and 15-30% of these individuals will develop Type 2 diabetes within 5 years.<sup>1</sup>

Type 2 diabetes is a progressive disorder; one goal is early detection of metabolic aberrations signifying the beginning of the pathology. Early identification provides an opportunity for lifestyle changes that can slow progression, however effective clinical tools for that detection are lacking. Insulin resistance is one early indicator, but currently there is not an effective clinical diagnostic tool for insulin resistance prior to the individual developing prediabetes. When glucose levels reach the diagnostic criteria for prediabetes, it is assumed insulin resistance is present.<sup>2</sup>

## 1.2 Glucose transporter 4 (GLUT-4): Cell Signaling, Trafficking, and Gene Regulation

Impaired glucose transporter 4 (GLUT-4) production and trafficking play a key role in the pathophysiology of type 2 diabetes and insulin resistance (IR). GLUT-4 has been a target for identification<sup>3,4</sup> and treatment<sup>5</sup> of glucose metabolism disorders. GLUT-4 resides in intracellular pools in membranes of small vesicles and is translocated to the cell membrane in response to insulin. Additionally, GLUT-4 has been shown to increase in skeletal muscle in humans and animals in response to exercise, independent of insulin.<sup>6-16</sup> It is commonly believed the increase in GLUT-4 levels is due to increased translocation from an intracellular pool rather than increased transcription, however that has not been conclusively determined.<sup>17</sup> Evidence supports the hypothesis that exercise stimulates a different mechanism and potentially draws from a different intracellular pool of GLUT-4.<sup>6</sup> Although individuals with IR or type 2 diabetes have diminished GLUT-4 response to insulin, the response to exercise may be uninhibited.<sup>6,10,18</sup> This is an important physiological consideration, as it provides a potential alternate pathway to improve blood glucose control in patients with IR.

Chronic exercise elevates basal GLUT-4 levels.<sup>8,12</sup> A GLUT-4 increase is seen both in conditioned athletes<sup>8</sup> and in individuals participating in shorter duration and less intense exercise.<sup>6,12,15</sup> Minimal strength training has similarly resulted in an increase in GLUT-4 in muscle tissue.<sup>10</sup> On the other hand, some studies have failed to show this increase in GLUT-4 in myocytes after short duration exercise.<sup>19,20</sup> As a whole, the data suggest that a variety of type, intensity, and duration of physical activity stimulate GLUT-4 translocation in skeletal muscle, improving cellular glucose transport.

The *SLC2A4* gene codes for the GLUT-4 protein and multiple transcriptional regulators are involved in *SLC2A4* expression. Exercise increases *SLC2A4* transcriptional activity.<sup>21-24</sup> Specific regulatory areas in the promoter region *SLC2A4* have been analyzed for their response to exercise. The myocyte enhancer factor (MEF2) shows increased binding in response to exercise.<sup>25-27</sup> Histone acetylation/deacetylation also appears to be involved in *SLC2A4* expression.<sup>27,28</sup> Overall, a better understanding of the impact of exercise on *SLC2A4* expression, GLUT-4 production, and GLUT-4 translocation is needed when considering interventions for glucose metabolism disorders.

### 1.3 Challenges in Researching GLUT-4

GLUT-4 is predominantly found in muscle and adipose tissue. One barrier to studying GLUT-4 regulation is the need for a biopsy from these tissues. The invasive nature and expense of this procedure has resulted in limited human studies and small sample sizes. Peripheral blood mononuclear cells (PBMC) are shown to have insulin-sensitive GLUT-4 activity and could potentially serve as a proxy tissue. Monocytes, a sub-population of mononuclear cells, were originally identified as having the majority of the insulin binding sites<sup>29,30</sup> as well as GLUT-4 activity<sup>31</sup> within the mononuclear cell population; therefore, monocytes have been used in many insulin sensitivity studies. Lymphocytes have also demonstrated GLUT-4 activity in response to insulin.<sup>3,5,32-34</sup> The research to date on GLUT-4 activity in mononuclear cells suggests these cells respond to insulin similar to muscle tissue. What has not been explored is whether they also respond to exercise in a similar fashion. Our lab recently reported, for the first time, higher GLUT-4 on mononuclear cells of conditioned versus sedentary sled dogs,<sup>35</sup> however similar findings have not been reported in humans.

Another challenge in diabetes prevention and treatment is the lack of early identification of those at risk. Early identification of individuals at risk for type 2 diabetes or IR may allow an exercise or lifestyle intervention to be implemented prior to disease progression. However, current routine diagnostic tools only detect physiological abnormalities once they result in inadequate glucose transport.<sup>4</sup> Inexpensive, direct, reliable identification of early physiological changes that are likely to lead to type 2 diabetes would be beneficial for early intervention.

#### 1.4 Study Purpose

The current study addressed the following research questions:

1) Do conditioned athletes have higher GLUT-4 levels in PBMC than sedentary individuals akin to the documented difference in skeletal muscle? Demonstrating an increase in PBMC GLUT-4 in conditioned individuals similar to that in myocytes would suggest that PBMC could provide a proxy tissue for studying GLUT-4 activity. The current study was designed as an initial exploration of this idea to determine whether more extensive studies are warranted.

2) Are there correlations between PBMC GLUT-4 and common indicators of insulin resistance? Correlations between PBMC GLUT-4 and common indicators of IR could lead to development of an early diagnostic tool for IR, promoting earlier interventions and prevention of disease progression.

3) Is there evidence for increased transcription of *SLC2A4* in PBMC in conditioned athletes versus sedentary individuals? Although much emphasis is placed on GLUT-4 translocation, *SLC2A4* transcription could also be an important molecular mechanism and a target for treatment. Again, PBMC may serve as a viable cell type for studying such the impact of diet, exercise, or medications on *SLC2A4* transcription.

4) Does dietary intake affect GLUT-4 or *SLC2A4* mRNA levels? Because nutrient intake is known to stimulate insulin, and thereby GLUT-4, dietary data was analyzed to assess relationships between specific nutrients and food groups and GLUT-4 activity in PBMCs.



## Chapter 2: GLUT-4 Regulation in Response to Exercise and Diet

### 2.1 Glucose Transporter 4 (GLUT-4) Protein

The solute carrier family 2 facilitated glucose transporter member 4 (GLUT-4) is a member of a family of facilitative glucose transporters. GLUT-4 is 509 amino acids long, with twelve helical transmembrane domains.<sup>36</sup> The protein is found in a variety of tissues, but most widely recognized in skeletal muscle and adipose tissue. GLUT-4 resides in storage vesicles (GSVs) within the cell, primarily in the perinuclear region.<sup>37</sup> Cell stimulation causes the GSV to translocate to the plasma membrane, delivering GLUT-4.<sup>38</sup> When cellular need for glucose diminishes, GLUT-4 is returned to the GSV. Insulin and exercise both have the capacity to trigger this process using distinct signaling pathways.<sup>39</sup>

#### 2.1.1 Stimulation of GLUT-4 Activity

Insulin is the most widely understood stimulator for GLUT-4 translocation. Insulin is released when there is an increase in nutrient supply, such as following a meal. Insulin binds to the insulin receptor on the plasma membrane which stimulates a complex signaling pathway involving insulin receptor substrate proteins (IRS), PI 3-kinase, phosphatidylinositol (3,4,5)P<sub>3</sub> (PIP<sub>3</sub>), and Akt/protein kinase B, among other intermediates.<sup>39,40</sup> This series of reactions promotes translocation of vesicles containing GLUT-4 to the plasma membrane, where they dock and fuse with the assistance of SNARE proteins.<sup>37</sup>

In addition to increased energy supply in the bloodstream from food sources, increased cellular energy demand triggers GLUT-4 translocation. Increased energy demand arises from stimuli such as muscle contraction or stress.<sup>37</sup> The exercise-stimulated pathway of GLUT-4



regulation is of interest in terms of a potential target for treatment of glucose metabolism disorders. This pathway has been examined as a potential target for ‘exercise mimetic’ pharmacological therapies.<sup>41</sup>

### 2.1.2 Exercise-Stimulation of GLUT-4 in Skeletal Muscle

Murine models provided early information on GLUT-4 content in skeletal muscle tissue in response to exercise. When compared to non-exercised control rats, both chronic and acute exercise elicit increases in muscle GLUT-4.<sup>13,14,42-44</sup> Exercise training increases GLUT-4 content in ranges from 30-95%.<sup>13,42</sup> Human studies have also demonstrated increased skeletal muscle GLUT-4 with acute<sup>9,11,21</sup> and chronic exercise.<sup>6,12</sup>

Since both acute and chronic exercise impact skeletal muscle GLUT-4 content, the amount of time between exercise and tissue sampling is a consideration. In human studies, an acute bout of exercise has been shown to increase plasma membrane GLUT-4 in as little as three hours following exercise<sup>22</sup> while others have shown a non-significant increase at eight hours leading to a significant increase at 22 hours post-exercise.<sup>9</sup> Leick et al., on the other hand, failed to detect a change in GLUT-4 at 10, 18, or 24 hours following an acute bout of exercise.<sup>21</sup> More regular exercise may increase the duration of GLUT-4 upregulation. When compared to their untrained counterparts, trained athletes show 93% higher GLUT-4 levels despite no training in the two days prior to muscle biopsy.<sup>8</sup> Following four weeks of cycling with one leg only, young men experience a 36% increase in GLUT-4 in the conditioned leg vs the non-conditioned leg 18 hours after the last exercise session.<sup>12</sup> Following two weeks of exercise, skeletal muscle GLUT-4 in young men has been shown to increase 26% over their pre-training levels when biopsies were taken 36-48 hours following the last exercise session.<sup>7</sup> It is difficult to draw clear conclusions

about the time frame in which skeletal muscle GLUT-4 begins to increase following exercise, how long the acute effect lasts, and how long any effect from regular physical activity persists. It does, however, appear that the increases begin fairly rapidly following exercise and persist for at least one to two days, particularly with a regular exercise protocol.

The specific fiber type of muscles being sampled may impact outcomes. Dagaard et al. demonstrated an increase in GLUT-4 in response to exercise in human muscle however when muscle-types were separated into slow-twitch or fast-twitch, only the slow-twitch muscles showed a response to exercise.<sup>7</sup>

Studies have demonstrated mixed results on the effects of exercise-stimulated GLUT-4 regulation in subjects with diabetes. After an acute bout of exercise, Kennedy et al. found similar increases in plasma membrane GLUT-4 in both diabetic and non-diabetic subjects.<sup>11</sup> Likewise, Christ-Roberts et al. showed subjects with and without diabetes had similar increases in both total and plasma membrane GLUT-4 following eight weeks of exercise.<sup>6</sup> Conversely, diabetic subjects undergoing strength training exercise show a 40% increase in GLUT-4 in their trained leg versus the non-trained leg whereas non-diabetic subjects show no change.<sup>10</sup>

## 2.2 Solute carrier family 2 member 4 (*SLC2A4*) gene

The human *SLC2A4* gene, also known as *GLUT-4*, is located on chromosome 17p13, is 6,314 base pairs long and codes for the GLUT-4 protein. *SLC2A4* activity is primarily reported in cardiac and skeletal muscle and adipocytes, however activity in other tissues and cells such as brain, kidney, pancreas, liver, and mononuclear cells have also been documented. The central role of GLUT-4 in blood glucose regulation, and therefore prevention and control of IR and type 2 diabetes, makes expression of *SLC2A4* of significant interest.

Single nucleotide polymorphisms (SNPs) can impact gene function. This poses the question of whether *SLC2A4* SNPs are associated with IR and/or type 2 diabetes. Early studies did not show differences in *SLC2A4* genotypes between individuals with versus without diabetes.<sup>45-50</sup> More recently, several SNPs have shown possible associations with type 2 diabetes and/or HbA1c levels including rs2654185<sup>51,52</sup>, rs5412<sup>52</sup>, rs5418<sup>51-53</sup>, rs5435<sup>52,53</sup>, and rs5421.<sup>53</sup> In some cases, multiple SNPs present simultaneously are required to see effects.<sup>52</sup> Despite these possible associations, research has primarily focused on *SLC2A4* transcription without regard to genotype.

### 2.2.1 *SLC2A4* Expression and Regulation

Individuals with type 2 diabetes do not necessarily have impaired *SLC2A4* expression or diminished levels of GLUT-4 however overexpression of the gene can improve glucose transport.<sup>40</sup> As such, an understanding of the molecular mechanisms of *SLC2A4* expression may provide insights for pharmacological or lifestyle interventions to improve glucose control.

The promoter region of genes is the primary site for the initiation of transcription. In the *SLC2A4* gene, several domains within 895 base pairs of the transcription start site have demonstrated importance in transcription.<sup>54</sup> One of the early regions of focus, that continues to be an active area of research, is the myocyte enhancer factor 2 (MEF2) binding region which binds multiple isoforms of MEF2.<sup>54-65</sup> Additional domains which have regulatory effects include an E-box (or hypoxia induced factor 1a, HIF1a) which is involved in MyoD activity,<sup>62,65</sup> a thyroid response element (TRE) which mediates the effects of thyroid hormones,<sup>62,66</sup> the so-called Domain I which binds GLUT-4 enhancer factor (GEF),<sup>58,63,64</sup> and an NF- $\kappa$ B binding site.<sup>67</sup>

The list of transcription factors and other regulatory molecules interacting with these domains continue to grow. MEF2A and MEF2D are essential, but not sufficient for *SLC2A4* transcription.<sup>57,68</sup> These two isoforms can exist as a heterodimer. MEF2A is diminished in insulin deficiency. A decrease in MEF2A is correlated to a reduction in GLUT-4 protein, pointing to a significant role for that particular isoform.<sup>68</sup> Despite the importance of MEF2A, other biomolecules are necessary for its full functionality. In muscle and cardiac cells, treatment with MyoD and TR $\alpha$ 1 along with MEF2A dramatically increase transcriptional activity.<sup>62</sup> Synergism between MEF2A and GEF in *SLC2A4* transcription in cultured cells has been demonstrated.<sup>56,58</sup> Weems et al. also note that both MEF2A and GEF are required for full activation of *SLC2A4* in adipocytes.<sup>55</sup> Michael et al. demonstrated that treatment of muscle cells in culture with PGC-1 proteins (transcriptional coactivators) improve basal glucose transport and increases GLUT-4 protein both in storage vesicles and on the plasma membrane.<sup>59</sup> Mutation of the MEF2 binding region ameliorates the increased levels suggesting the effects are a result of increased gene expression. Gene-gene interaction may impact *SLC2A4* expression. Recently, interaction between *SLC2A4* and zinc finger protein 407 (*Zfp407*) has been identified. Knockdown of *Zfp407* reduces *SLC2A4* mRNA, GLUT-4 protein, and insulin-stimulated glucose uptake.

Histones package DNA and play a role in gene expression by mediating chromatin remodeling such that transcription factors have more or less ability to bind to a gene. Histones can be modified by acetylation, methylation, ubiquitination, sumoylation, phosphorylation, glycosylation, and ADP ribosylation, however the most common modifications are acetylation and methylation.<sup>69</sup> Generally, hyperacetylation causes chromatin to have a more open structure, allowing transcription factors access to binding sites and thus increase gene expression; hypoacetylation has the opposite effect. There are two primary categories of enzymes involved in

histone acetylation: histone deacetylases (HDACs) and histone acetyltransferases (HATs).

HDACs decrease acetylation and HATs increase acetylation.

The role of histone acetylation has been examined in regard to *SLC2A4* transcription. There is an inverse relationship between nuclear HDAC5 levels and the expression of GLUT-4.<sup>55,63</sup> HDAC5 knockdown in mouse and human cell lines increases *SLC2A4* expression and basal glucose uptake.<sup>70</sup> HDAC5 has been shown to complex with MEF2A and GEF and contribute to decreased gene expression even in the presence of these transcription factors, supporting a regulatory role for HDAC5.<sup>63</sup>

Several metabolic signaling systems have been examined for their role in GLUT-4 regulation including  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (CaMK), calcineurin, and 5' AMP-activated protein kinase (AMPK). Calcium ( $\text{Ca}^{2+}$ ) release from the sarcoplasmic reticulum triggers many signaling cascades. Calmodulin is a ubiquitously expressed protein that binds  $\text{Ca}^{2+}$ , allowing it to interact with other molecules. The  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMK II) facilitates phosphate transfer between molecules including from ATP to substrates and thus is key in energy systems. Calcineurin is also a serine/threonine phosphatase involved in dephosphorylation which alters a molecule's activity. 5' AMP activated protein kinase (AMPK) is a regulator of cellular metabolism and therefore important in muscle tissue. When energy needs increase, AMPK promotes fatty acid oxidation and glucose mobilization. Therefore CaMK, AMPK, and calcineurin have potential roles in GLUT-4 regulation.

AICAR is a pharmacological method used in research to stimulate AMPK activity. The muscle tissues of mice and rats treated with AICAR have increased levels of GLUT-4 protein<sup>64</sup> and *SLC2A4* mRNA.<sup>64,71</sup> These changes may happen in a fiber-dependent manner.<sup>64</sup> Zeng et al. found both the protein and mRNA levels were highest in white fibers of the quadriceps (primarily

type IIb), elevated in red fibers (primarily IIa), but not elevated in soleus (primarily type I fibers).<sup>64</sup> It is noteworthy that these changes are time dependent, peaking and returning to normal at 13 hours and 24 hours after treatment, respectively.

Evidence supports that the AMPK and CaMK pathways play a role in histone deacetylation via HDACs, impacting *SLC2A4* regulation. In muscle tissue from the vastus lateralis muscle in men, McGee et al. demonstrated AMPK phosphorylates HDAC5 on Ser259 and Ser498; HDAC5 is subsequently exported from the nucleus allowing increased acetylation of the MEF2 binding region triggering increased binding of MEF2A, which results in increased *SLC2A4* mRNA.<sup>54</sup> The authors concluded that HDAC5 is a repressor of *SLC2A4* expression via HDAC5 phosphorylation by AMPK in skeletal muscle. Mukwevho et al. used caffeine to stimulate  $\text{Ca}^{2+}$  release in cultured myocytes.<sup>60</sup> Stimulation with caffeine causes increased *SLC2A4* mRNA, but that effect is abolished with administration of dantrolene (an inhibitor of calcium release from the sarcoplasmic reticulum), supporting the role of  $\text{Ca}^{2+}$  in the signaling cascade. Administration of caffeine causes export of HDAC5 from the nucleus, acetylation of histone H3, and increased MEF2A binding (no effect on MEF2A quantity), but again, all effects are attenuated by dantrolene. In mice, inhibiting the calcineurin, CaMK, or AMPK pathway reduces, but does not eliminate, *SLC2A4* promoter activity in slow-twitch muscle fibers.<sup>61</sup> The calcineurin pathway appears to dominate in fast-twitch fibers.<sup>61</sup>

## 2.2.2 Exercise-induced *SLC2A4* Regulation in Skeletal Muscle

Exercise has been shown not only to increase GLUT-4 translocation to the cell surface of myocytes, but also to upregulate *SLC2A4* in humans, murine models, and cell culture. In murine models, both acute exercise<sup>25,26,42,72-74</sup> and chronic training<sup>27,43</sup> have increased *SLC2A4* mRNA.

Other markers of transcription have shown similar results.<sup>75</sup> The upregulation appears to be rapid, with mRNA increasing within the first six hours upon cessation of exercise<sup>25,26,75</sup> and maintaining elevation for at least 12 hours following exercise.<sup>25-27,42,72,73</sup> It is noteworthy that the exercise protocol of many of these animal studies consists of six hours of exercise on a single day<sup>43,72,73,76</sup> however six hours per day for five days<sup>43</sup> or a more modest 60 minutes per day for 28 days<sup>27</sup> all resulted in increases in *SLC2A4* mRNA.

Human studies have shown increased *SLC2A4* mRNA immediately following a single, hour-long bout of exercise<sup>22,23,77</sup> and three hours following such exercise.<sup>22-24</sup> Ninety minutes of exercise results in elevated mRNA at ten hours following cessation of exercise,<sup>21</sup> however at eighteen<sup>21</sup> to twenty-four<sup>15,21</sup> hours following either sixty or ninety minutes of acute exercise, no significant changes from baseline are detected. Continuing the sixty minutes per day of exercise for seven days also does not cause mRNA to be elevated at 24 hours following completion of the final exercise session.<sup>15</sup>

The transcription factors and signaling pathways involved in *SLC2A4* regulation in response to exercise continue to be investigated. Murine models have demonstrated increased MEF2A binding in response to exercise.<sup>25-27</sup> Some studies demonstrate an increase in total MEF2A protein<sup>43</sup> or *MEF2A* mRNA<sup>74</sup> while others show increased binding, but no increase in total protein content<sup>25,26</sup> suggesting exercise increases translocation rather than gene upregulation. Similarly, in humans, both MEF2A and MEF2D show increased binding immediately following a single bout of exercise and nuclear MEF2A, but not MEF2D, increase following the exercise.<sup>78</sup> In murine models, CaMK II increases immediately following exercise<sup>25</sup> and blocking CaMK II activity abolishes the increased MEF2A binding.<sup>26</sup> In mice, overexpression of AMPK<sub>2α</sub> increases MEF2A binding; knockout of AMPK<sub>2α</sub> reduces, but does

not eliminate binding.<sup>27</sup> Another study in mice with a mutated AMPK  $\alpha$ -subunit did not show any difference in *SLC2A4* mRNA following exercise compared to wild-type mice.<sup>73</sup> Inhibiting calcineurin also has no effect on MEF2A protein content in rats.<sup>43</sup> Human data also suggest that the calcineurin pathway is not responsible for *SLC2A4* activation in response to exercise.<sup>77</sup> These data support that exercise increases MEF2A binding to the promoter region of the *SLC2A4* gene; CaMK II is involved in the signaling for binding and AMPK<sub>2 $\alpha$</sub>  may play a role, but is not exclusively responsible. It is noteworthy, however that there may be some variability in pathways depending on predominant muscle fiber type in tissues sampled.<sup>79</sup>

A more general look at binding sites and transcripts in murine models have shown increased MEF2A, MEF2D, HIF1 $\alpha$  mRNA in response to contraction.<sup>74</sup> Additionally, increased binding activity has been observed at the MEF2 (AT rich element), E-box (HIF1- $\alpha$ ),<sup>74,80</sup> and TR $\alpha$ <sup>80</sup> sites on the gene. Although NF- $\kappa$ B appears to have a role in insulin-stimulated *SLC2A4* regulation, exercise does not increase NF- $\kappa$ B binding in rat skeletal muscle.<sup>74</sup> Human studies demonstrate an immediate increase in GEF binding<sup>78</sup> and MyoD, myogenin, and MRF4 upregulation<sup>28</sup> in response to a single bout of exercise.

Histone acetylation has been examined for its relationship with *SLC2A4* expression. Following sixty minutes of cycling, male subjects show no change in total HDAC5 in skeletal muscle, but a decrease in nuclear HDAC5 and MEF2 associated HDAC5.<sup>77</sup> Increased phosphorylation of HDAC5 at Ser498 is seen after 90 minutes of cycling.<sup>28</sup> Similarly, mice show decreased nuclear, but not total, HDAC5 following 28 days of an exercise protocol.<sup>27</sup> It appears that, in skeletal muscle, exercise stimulates phosphorylation of HDAC5 causing it to translocate from the nucleus, creating exposure of the MEF2 binding site on the *SLC2A4* gene, allowing binding of transcription factors.



Most human studies on GLUT-4 in skeletal tissue in response to exercise have utilized young, healthy participants, primarily male. This may or may not impact outcomes however caution should be used when applying these results to other populations. Conditioning level may impact *SLC2A4* transcription and GLUT-4 activity. A study of 98 twin pairs showed a positive correlation between  $\text{VO}_2$  max and *SLC2A4* mRNA.<sup>81</sup> Hussey et al. failed to detect differences in *SLC2A4* mRNA response to exercise in patients with and without type 2 diabetes.<sup>23</sup> Limited studies have assessed the role of sex in *SLC2A4* gene expression; however, Storgaard et al. observed elevated *SLC2A4* mRNA in men compared to women.<sup>81</sup> Vissing et al. noted differences in MEF2A and MEF2D expression in men and women, but other regulatory factors were similar between sexes.<sup>28</sup>

### 2.3 Relationship of Nutrition and GLUT-4 Regulation

The impact of various nutritional factors on GLUT-4 regulation in a wide range of cells including cardiac,<sup>82</sup> renal,<sup>83</sup> adipose,<sup>84</sup> and skeletal muscle<sup>85</sup> cells have been investigated. A “Western diet” which is high in saturated fat and simple carbohydrates is known to have negative metabolic effects. Mice given a Western diet for 16 weeks have no difference in total cellular GLUT-4 in cardiac cells than control mice receiving a standard diet, but have decreased plasma membrane GLUT-4.<sup>82</sup> This suggests decreased translocation and therefore inefficiency of GLUT-4. The macronutrient distribution of the diet could potentially impact GLUT-4 expression. Most human studies on skeletal muscle GLUT-4 changes in response to exercise have failed to account for diet. Studies which have provided a standardized diet have varied from a very high carbohydrate diet (~75% carbohydrates, 15% protein, and 10% fat)<sup>15,22</sup> to a modest carbohydrate diet (53% carbohydrate, 17% protein, 30% fat).<sup>21</sup> These studies yielded different

results despite similar participant demographics and exercise protocols. However the time interval following exercise that muscle samples were collected were different, therefore it is difficult to assess whether the time factor accounts for different results or whether there is another contributing factor, such as carbohydrate intake.

Dietary fat intake, and specifically the type of fat, has been investigated for its relationship to insulin resistance. Adipocytes incubated with arachidonic acid (AA), an omega-6 polyunsaturated fatty acid, show increased GLUT-4 protein on the cell membrane without an increase in total cell GLUT-4 protein suggesting that AA stimulates GLUT-4 translocation.<sup>84</sup> This increased GLUT-4 corresponds with increased cellular glucose uptake. Human podocytes in cell culture show decreased GLUT-4 translocation in response to insulin and glucose when the cells are incubated with palmitate, a saturated fatty acid.<sup>83</sup> Human cultured skeletal muscle cells show increased *SLC2A4* gene expression when conjugated linoleic acid (CLA), an omega-6 fatty acid, or a combination of the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are given.<sup>85</sup> There was increased glycolytic capacity specifically with the omega-3 combination treatment. Cultured skeletal cells incubated with either oleic acid (an omega-9 monounsaturated fatty acid) or linoleic acid (an omega-6 polyunsaturated fatty acid) show a decrease in both *SLC2A4* mRNA and GLUT-4 protein compared to untreated cells.<sup>86</sup>

Bioactive compounds, often in the form of dietary supplements, have been of interest due to availability, consumer interest, and potential potency. Creatine is one such supplement that has been heavily researched for its role in exercise. Female rats given creatine, in addition to their regular chow, have increased GLUT-4 protein and *SLC2A4* mRNA in skeletal muscle compared to rats receiving a placebo.<sup>87</sup> The creatine group also exhibit increased nuclear MEF2A and MEF2D as well as MEF2 binding. Extracts from *Momordica charntia*, a plant native to semi-

tropical climates, given to myocytes in cell culture increases *SLC2A4* mRNA 3.6-fold which is similar to effects by insulin and rosiglitazone.<sup>88</sup> Blocking protein synthesis negates the effects, supporting a role of additional protein production as necessary for upregulation. Resveratrol is another bioactive, found in the skin of grapes among other sources, which has been studied for its antioxidant and other health-related properties. Chinese men with type 2 diabetes, taking a 500 mg daily resveratrol supplement for 12 weeks show a trend toward increased expression of GLUT-4 in muscle cells compared to controls taking a placebo.<sup>89</sup> The sample size of five per group may have been too small to detect significant changes. The activity level of those taking the resveratrol was also lower (by chance, not design) which could have modestly attenuated a potential effect of the treatment on GLUT-4 levels. It is nonetheless a noteworthy trend for such a small sample. These data give reason to consider both food intake and dietary supplements as a potential mediator of GLUT-4.

Maternal prenatal diet is known to impact gene expression in offspring, potentially affecting multiple generations. Animal models are necessarily used for much of this type of research. Female rats born to mothers who were restricted calories during pregnancy to induce intrauterine growth retardation (IUGR) have decreased *SLC2A4* mRNA in skeletal muscle as adults; this is not seen in male offspring.<sup>90</sup> The offspring of IUGR dams also have decreased MEF2 binding (without a change in MEF2 protein quantity), decreased MyoD binding, and decreased histone H3 acetylation. Zheng et al. assessed the impacts of maternal protein restriction on GLUT-4 expression and found female offspring have decreased GLUT-4 protein and *SLC2A4* mRNA, but there no difference in male offspring. Additionally, the female offspring have increased MEF2 protein and H3 and H4 acetylation and, again, no change in males.<sup>91</sup> Increased glucocorticoid exposure during pregnancy in rats has elicited a 15-fold

increase in *SLC2A4* mRNA in skeletal muscle of the offspring.<sup>92</sup> This results in an increase in intracellular GLUT-4 protein, however no increase in plasma membrane GLUT-4. A high omega-3 fatty acid diet was provided in an attempt to attenuate the effects of the glucocorticoids, but no difference was seen. These studies support, not only that prenatal nutrition and environment may be an important regulator of GLUT-4 expression, but this may occur in a sex-specific manner.

The upregulation of *SLC2A4* in response to exercise may be attenuated by dietary factors, specifically carbohydrates. Human, male subjects undergoing an acute bout of exercise show increased *SLC2A4* mRNA in skeletal muscle cells as would be expected, however when they ingested a high glucose beverage during exercise and recovery there is a non-significant, but noteworthy attenuation of this exercise-stimulated upregulation.<sup>24</sup> Rats subjected to six days of exercise training show increased GLUT-4 protein, increased MEF2A binding to *SLC2A4*, increased histone H3 acetylation, decreased nuclear HDAC5, and increased pAMPK/AMPK in skeletal muscle cells, all of which are consistent with other exercise studies.<sup>93</sup> However, some groups of rats in this study were provided access to either a high fructose beverage, a high maltodextrin beverage, or plain water (in addition to standard rat chow). Both of the sugar beverages attenuated the exercise-induced increases in GLUT-4, MEF2A binding, and histone H3 acetylation; the fructose beverage attenuated the phosphorylation of AMPK and the decrease in HDAC5. These studies suggest that simple sugars may negate some of the beneficial effects of exercise in terms of GLUT-4 regulation.

Nutritional intake may indeed impact GLUT-4 regulation in a variety of cell types independently, or more likely, interacting with other modulating mechanisms. Specific types of

fats, sugars, and other bioactive compounds as well as overall dietary patterns warrant investigation.

## 2.4 GLUT-4 in Peripheral Blood Mononuclear Cells (PBMC)

As long as four decades ago, it was recognized that human PBMCs contain insulin receptors and bind insulin in a quantity relative to the number of monocytes.<sup>94</sup> Monocytes were found to be the major subpopulation of PBMCs to bind insulin, accounting for approximately 80% of insulin binding activity within PBMCs.<sup>29</sup> More recently, lymphocytes have shown to be responsive to insulin as well.<sup>34</sup> GLUT-4 is present in PBMCs.<sup>17</sup> Similar to insulin binding activity, monocytes appear to have the most GLUT-4 activity among PBMCs.<sup>31,34,95</sup> Monocytes treated with insulin increase GLUT-4 on the plasma membrane from 24-54%.<sup>96-98</sup>

The impact of obesity and insulin resistance disorders such as type 2 diabetes appear to decrease insulin binding to monocytes similar to what is observed in myocytes and adipocytes.<sup>99,100</sup> As would be predicted based on activity in other cell types, glucose clearance is increased with increased insulin binding.<sup>100</sup> A reduced quantity of GLUT-4 has been seen in monocytes of diabetic patients compared to healthy controls however the *SLC2A4* mRNA is no different between the groups.<sup>101</sup> Lymphocytes also show GLUT-4 activity similar to myocytes and adipocytes. Subjects with pre-diabetes have decreased lymphocyte GLUT-4 levels compared to their euglycemic peers.<sup>3</sup> Interestingly, the GLUT-4 levels of euglycemic subjects with a family history of type 2 diabetes mimic their pre-diabetic counterparts.<sup>3</sup> This pattern would fit with the pathophysiology of diabetes whereby insulin levels increase in the early stages attempting to compensate for slight elevations in blood glucose. The increased insulin stimulates

increased GLUT-4 activity. The interesting finding is that this pattern is observed in PBMCs similar to other insulin-sensitive tissues.

Despite decades of data demonstrating that mononuclear cells express insulin-stimulated GLUT-4 activity, research has not focused on exercise-stimulated GLUT-4 activity in this cell population. PBMCs are an attractive cell type for such research due to their ease of accessibility for study as well as their role in immunity and inflammation, which is now a well-understood component of metabolic disorders.



## Chapter 3: Materials and Methods

### 3.1 Study Design

This study used a cross-sectional design of participants who were students at the University of Alaska Fairbanks (UAF) at the time of the study.

#### 3.1.1 IRB and Consent

The study protocol was approved by the Institutional Review Board of the University of Alaska Fairbanks (#492213-4) and acknowledged by the Institutional Review Board of the University of Alaska Anchorage (Appendix A). Written consent was obtained prior to beginning data collection.

#### 3.1.2 Participants

All participants were students at UAF, between 18 and 25 years of age, non-pregnant, and non-diabetic. The sample consisted of two groups: conditioned athletes and sedentary students. Conditioned participants were recruited through the UAF cross-country skiing and cross-country running teams. This group consisted of endurance athletes who had been training for ten to twenty hours per week for three months prior to sample collection. Exercise training involved both team and individual plans, but was designed to prepare athletes for competition in cross-country running and/or cross-country skiing between September and March. Sedentary participants were recruited through a variety of ways including speaking to introductory level science classes, posting flyers around campus, and through social media such as UAF's Facebook and Pinterest pages. Sedentary students did not participate in regular moderate



physical activity (defined to participants as “physical activity that takes moderate effort and makes you breathe somewhat harder than normal”) for more than 20 minutes one time per week over the past three months. Sedentary students were pre-screened using a questionnaire created and administered through google forms.

Participants completed a health history questionnaire which included demographic information such as age, sex, and ethnicity (Appendix B). The health history confirmed there was no known history of diabetes. Participants were 90% white.

### 3.2 Dietary, Physical Activity, and Biometric Assessment

Dietary and physical activity data and body measurements were collected at one time during the study. Participants were instructed to record their dietary intake as described below and complete the physical activity questionnaire prior to presenting to the clinic for the biometric assessment and blood draw. Participants presented to the clinic in the morning following an overnight fast.

#### 3.2.1 Dietary Assessment

Dietary data was collected using the ASA24 Automated Self-Administered 24-hour Recall system from the National Cancer Institute. The ASA24 is a computer-based system modeled after the USDA Automated Multiple Pass Method (AMPM) which is used in the National Health and Nutrition Examination Survey (NHANES). The ASA24 has been shown to have acceptable performance in measuring true intake and is comparable to an interviewer-administered 24-hour recall in populations such as the one sampled for this study.<sup>102,103</sup> Participants were instructed on the tool following informed consent. They were asked to record

one typical weekend day and one typical weekday of food intake. Dietary data was reviewed and cleaned according to ASA24 recommendations. Specific nutrients and food groups were analyzed for similarities between the two groups and for any association with GLUT-4 protein or *SLC2A4* gene expression. Fruit and vegetable intake, total calories, macronutrient distribution, sugar, fiber, saturated fat, arachidonic acid, palmitate, oleic acid, and linoleic acid were analyzed.

### 3.2.2 Physical Activity Assessment

Participants completed the International Physical Activity Questionnaire (IPAQ) Short Form with instructions from a researcher (Appendix C). The IPAQ has been assessed for validity and reliability in multiple populations.<sup>104</sup> The data in the questionnaires were analyzed and cleaned according to the IPAQ Guidelines for Data Processing and Analysis. MET-minute/week scores were calculated and participants were classified categorically according to the protocol as low, moderate, or high physical activity levels.

### 3.2.3 Biometric Assessment

A Registered Nurse with the Center for Alaska Native Health Research performed anthropometric measurements. Height was measured to the nearest 1/8 inch. Weight in pounds and percent body fat were measured with a TANITA TBF-300A (Tanita Corporation of America Inc., Arlington Hills, Illinois); shoes, socks, and heavy clothing were removed. The “standard” setting on the TANITA was utilized regardless of physical activity level for consistency in measurements. Waist circumference was measured with the Gulick II 150 cm anthropometric tape. Two measurements to the nearest 0.2 cm were obtained and the average was utilized in the analysis. If measurements differed by more than two cm an additional measurement was taken.

### 3.3 Laboratory Assessment

Participants were instructed to fast for 12 hours prior to the blood draw with nothing consumed except water. Blood was obtained by venipuncture into one 8-ml BD separation tube, one 4-ml EDTA tube, and one 2-ml heparinized tube. Samples not immediately used were refrigerated or centrifuged within two hours of sample collection. Plasma from heparinized tubes was frozen at -80° C for future measurement of insulin.

#### 3.3.1 Metabolic Parameters

Blood lipids, glucose, and HbA1c were measured immediately following each blood draw utilizing blood from the heparinized tube at UAF's Center for Alaska Native Health Research clinic. The Cholestech LDX system measured total cholesterol, LDL, HDL, triglycerides, and glucose; the Bayer DCA 2000+ Analyzer measured HbA1c.

Plasma insulin was measured using a commercially available ELISA kit. Tests were run in duplicate. The Insulin ELISA (ALPCO Immunoassays, United States) kit was used according to manufacturer's instructions; absorbance was read at 450 nm. Absorbance readings were collected using a Synergy HT multi-mode microplate reader (BioTek, United States).

The homeostasis model assessment of insulin resistance (HOMA-IR) has been widely used as a surrogate for assessing insulin resistance however universal cut-off values have not been defined.<sup>105-108</sup> HOMA-IR was calculated with the equation (fasting plasma insulin \* fasting plasma glucose)/22.5.<sup>105-108</sup>

### 3.3.2 GLUT-4 Protein

The BD separation tubes were centrifuged at 3600 RPM for 15 minutes at room temperature. The mononuclear cell layer was then collected and transferred to a 15-mL conical vial. The sample was re-suspended in 15 mL RPMI 1640. Tubes were centrifuged for 15 minutes at 1500 RPM. Samples were washed 2 times and centrifuged for 15 minutes at 1500 RPM after each wash. The final sample was re-suspended with RPMI 1640 to a final volume of 8 mL from which the sample for the GLUT-4 protein analysis was drawn.

The GLUT-4 protein was measured using a commercially available ELISA kit. Tests were run in duplicate. The Glucose Transporter 4 (GLUT-4) kit (USCN Life Sciences, Inc., United States) kit was used according to manufacturer's instructions; absorbance was read at 450 nm. Absorbance readings were collected using a Synergy HT multi-mode microplate reader (BioTek, United States).

### 3.3.3 Genomic Labs

The genomic lab experiments were designed to assess differences in MEF2 and HDAC5 binding to the *SLC2A4* promoter region and relative quantities of *SLC2A4* mRNA between the conditioned and sedentary groups. The goal was to determine whether patterns were similar to what has been observed in myocytes.

*Chromatin Immunoprecipitation.* Chromatin was isolated from the mononuclear cells with a commercial chromatin extraction kit (Abcam Episeeker Chromatin Extraction Kit). The cells from approximately 3 ml of whole blood were utilized. The BD separation tubes yield approximately 1.3 million PBMCs per 1 ml whole blood. This value was used to estimate cell quantity for experiments. The manufacturer's protocol for suspension cells was followed

assuming approximately  $3 \times 10^6$  cells. The protocol estimated a yield of 4  $\mu\text{g}$  of chromatin per  $10^6$  cells, therefore it was estimated that the current chromatin extraction yielded approximately 12  $\mu\text{g}$  of chromatin.

The estimated 12  $\mu\text{g}$  of chromatin was utilized for chromatin immunoprecipitation with a commercial ChIP kit (AbCam EpiSeeker ChIP Kit – One Step). MEF2 and HDAC5 were immunoprecipitated using commercially available antibodies (Santa Cruz Biotechnolog, Inc). The manufacturer's protocol was followed for the ChIP reaction using RNA polymerase II as a positive control and IgG as a negative control. Concentrations were quantified with a nanodrop machine to move forward with real time RT-PCR. Unfortunately, there were undetectable quantities of DNA in the samples, therefore the subsequent planned experiment to analyze quantities of MEF2 and HDAC5 binding to the promoter region of *SLC2A4* was not carried out.

*SLC2A4 mRNA*. RNA was extracted from the mononuclear cells from approximately 3 ml of whole blood (with an estimated 1.3 million cells per ml of whole blood as previously described). The TRIzol (Life Technologies) reagent and protocol was utilized. The interphase containing protein and DNA was stored at  $-80^\circ\text{C}$  for later analysis. Extracted RNA was quantified using the nanodrop machine. cDNA was synthesized using First Strand cDNA Synthesis protocol (Life Technologies). The RNA volume added to each reaction was adjusted based on the concentration of RNA in the sample such that 1  $\mu\text{g}$  RNA was utilized for each cDNA synthesis reaction.

The real time RT-PCR experiment was set up with 1  $\mu\text{l}$  of cDNA in each reaction. Reactions were run in triplicate. *GAPDH* was used as an internal control (“housekeeper gene”).<sup>109,110</sup> Real time RT-PCR was performed using SYBR green chemistry on the Step One Plus RT PCR System (Applied Biosystems). Primers were designed to amplify the MEF2

binding region of *SLC2A4* (forward: 5'-CCT GAC ATT TGG AGG CTC-3'; reverse: 5'-GGA GCA ATG CCC CAA AG-3').<sup>54</sup> Forty cycles of PCR were utilized for amplification.

Experiments with a standard deviation between triplicates of greater than 1.0 were repeated until acceptable standard deviations were achieved. Relative quantification was conducted by expressing the cycle threshold ( $C_T$ ) values for *SLC2A4* relative to the GAPDH internal control. As will be presented in the results section, GAPDH did not show consistent expression between the two groups in this study, despite literature that suggests it is an appropriate internal control for *SLC2A4*.<sup>109,110</sup> Because of this, the log transformation of the actual  $C_T$  ( $2^{-C_T}$ ) was used for further analysis.

*GLUT-4 Enhancer Factor (GEF) and GLUT-4 Protein Analysis.* The TRIzol protocol was utilized to isolate protein from the interphase saved from the RNA isolation described above. Protein was re-suspended in SDS according to protocol and stored at -20° C until protein quantification. Protein was quantified using the Lowry protocol with the *RC DC*<sup>TM</sup> (reducing agent and detergent compatible) Protein Assay (Bio Rad) using 25 µl of sample.

SDS-PAGE and immunoblotting were performed using standard techniques. A 10% SDS-PAGE gel was utilized. The quantity of sample loaded onto the gel was calculated based on the initial protein concentration to provide a loading concentration of 0.8 mg/ml in a total volume of 20 µl of loading solution. The protein yield of many samples in the initial batches of experiments was very low. When the stock of isopropanol was replaced, the subsequent experiments had a visually identifiable difference in the protein pellet following that step and a more expected protein yield which led to the conclusion that the isopropanol used for the initial batches of experiments was most likely the problem in the initial experiments. For this reason, only nine samples were used for the remaining protein experiments. Even of the nine remaining

samples, protein concentrations of some were limited; 0.8 mg/ml was selected as the highest concentration that could be obtained from all remaining samples. After SDS-PAGE, immunoblotting was done with anti-glucose transporter GLUT-4 antibody (Abcam) and anti-SLC2A4RG antibody (Abcam). Membranes were incubated with primary antibodies overnight at 4°C with gentle shaking. Secondary antibodies were species-specific (anti-rabbit antibody for anti-SLC2A4RG/GEF and anti-mouse for anti-GLUT4) horseradish peroxides-conjugated immunoglobulins. Multiple concentrations of primary and secondary antibodies were used in an attempt to optimize the Western blot (See Appendix D for details of attempts at experiment optimization). SuperSignal™ West Femto Maximum Sensitivity Substrate (Life Technologies) was used for chemiluminescent imaging.

### 3.4 Statistical analysis

SPSS statistical analysis software (version 21) was used to analyze the data. Student's independent sample t-tests were used to assess differences between the conditioned and sedentary groups (differences were considered significance at  $\alpha \leq 0.05$ ) related to demographic, anthropometric, biochemical, and dietary data. Student's independent t-tests were also used to assess differences in GLUT-4 and *SLC2A4* between males and females. The SPSS box-plot was used to assess outliers, the Shapiro-Wilk test to assess normal distribution of the data, and Levene's test to assess homogeneity of variances prior to conducting the t-test. The Mann-Whitney U test was used to assess physical activity data between the groups and non-parametric data. Pearson's correlation coefficient or Spearman's rank correlation coefficient on the combined groups were used to investigate associations between GLUT-4 and glucose, HbA1c, insulin, HOMA-IR, BMI, percent body fat, and dietary factors.

## Chapter 4: Results

### 4.1 Demographics

Conditioned participants had a mean age of  $20.1 \pm 2.0$  years and the sedentary participants had a mean age of  $21.5 \pm 2.1$  years. There were no significant differences between the groups in regard to age or sex (Table 1).

**TABLE 1: Demographics**

Parameter	Conditioned	Sedentary
n	16	15
Age (years)	$20.1 \pm 2.0$	$21.5 \pm 2.1$
Sex:		
<i>Male</i>	8	8
<i>Female</i>	8	7

Demographic data as reported on health history form.

Data are means  $\pm$  standard deviations.

No significant differences between groups for age or sex.

### 4.2 Diet, Physical Activity, and Biometric Assessment

Diet intake, physical activity, and body measurements were assessed. These indicators were analyzed for similarities and/or differences between groups as well as correlations with GLUT-4.



#### 4.2.1 Diet Assessment

Dietary factors related to blood glucose regulation were analyzed (Table 2). There was no significant difference in macronutrient distribution between the two groups. The conditioned group had a significantly higher total caloric intake ( $p=0.004$ ), sugar intake ( $p=0.002$ ), fiber intake ( $p<0.0001$ ), and fruit and vegetable intake ( $p=0.005$ ) than the sedentary group. When sugar, fiber, and fruit and vegetable intake were normalized to individual caloric intake, there was found to be no significant difference in mean grams of sugar per calorie between the groups ( $p=0.461$ ). Servings of fruit and vegetables and grams of fiber intake per calorie were non-normally distributed therefore mean ranks were analyzed using a Mann-Whitney U test. The conditioned group had a higher mean rank of gram of fiber per calorie intake (19.97) than the sedentary group (11.77),  $U = 56.500$ ,  $z = -2.551$ ,  $p=0.011$ . The conditioned group also had a higher mean rank of servings of fruits and vegetables per calorie intake (19.25) than the sedentary group (12.3),  $U=68.000$ ,  $z = -2.055$ ,  $p=0.041$ . Using Pearson's correlation coefficient, no associations between GLUT-4 and calories, sugar, fiber, or fruit and vegetable intake could be detected.

**TABLE 2: Calorie, Macronutrient, and Fruit/Vegetable Intake**

Parameter	Conditioned	Sedentary	p-value
	Mean	Mean	
Fruit and Vegetable Intake (servings/day)	3.8 (2.2)	1.9 (1.1)	0.005*
Caloric Intake (kcal/day)	3022 (951)	2041 (756)	0.004*
Calories from Carbohydrate (%)	51.7 (5.1)	47.5 (6.8)	0.060

**TABLE 2 Cont.**

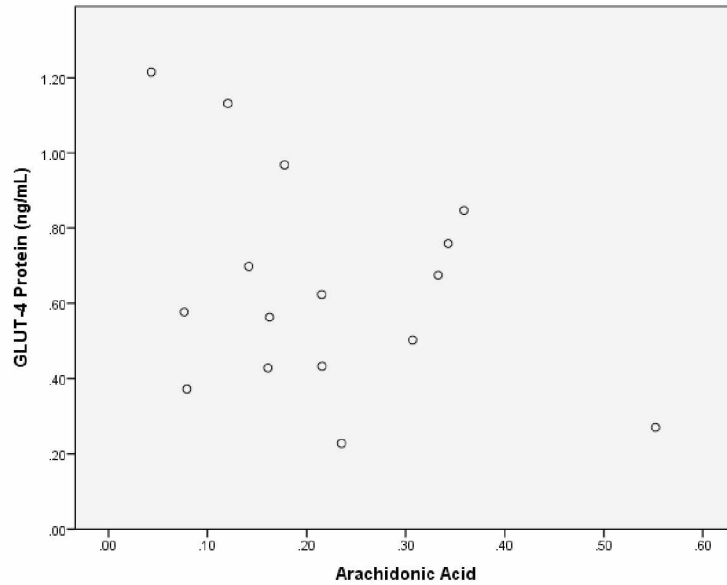
Calories from Fat (%)	34.5 (3.9)	35.6 (5.2)	0.488
Calories from Protein (%)	15.4 (2.1)	14.3 (2.7)	0.223
Sugar Intake (g/day)	171.5 (63.2)	104.7 (46.0)	0.002*
Fiber Intake (g/day)	29.0 (11.2)	14.2 (7.5)	< 0.0001*

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Dietary data as assessed by ASA24 program. Data are reported as means (standard deviations).

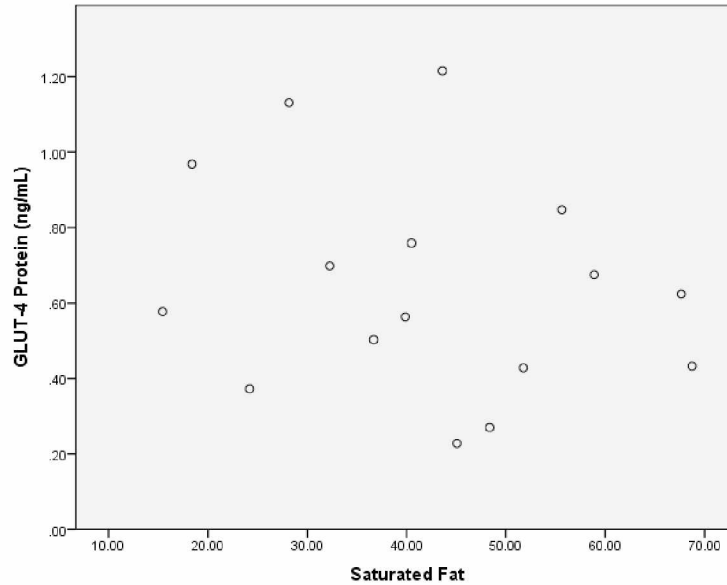
\* significant at  $p \leq 0.05$

Based on findings of other studies,<sup>83,84,86</sup> correlations between specific types of fat and GLUT-4 were analyzed. For both groups combined, a scatterplot showed no relationship between GLUT-4 and saturated fat, arachidonic acid, palmitate, oleic acid, or linoleic acid (Figures 1-5). The intake of these fats within the conditioned group was normally distributed, however in the sedentary group only arachidonic acid and oleic acid were normally distributed. For this reason, and to control for conditioning, the conditioned group was analyzed separately as well. Still, no associations between these fatty acids and GLUT-4 could be detected.



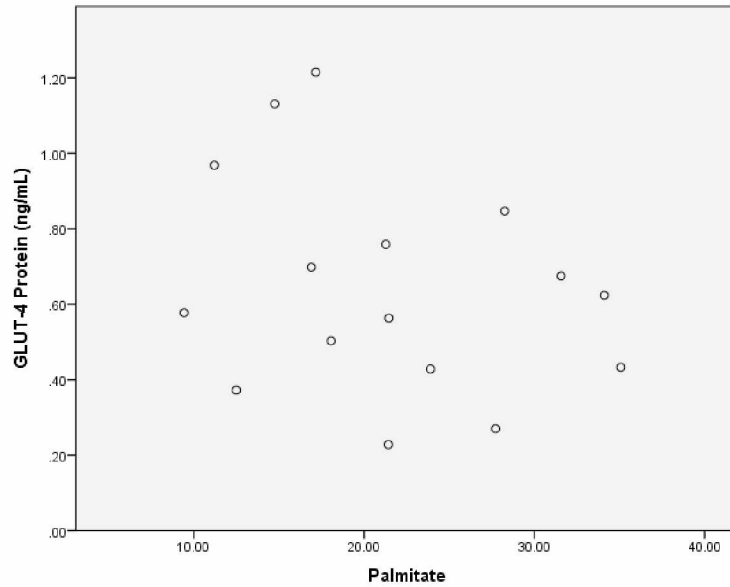
**FIGURE 1: Relationship Between GLUT-4 Protein and Arachidonic Acid Intake**

There is no association between GLUT-4 protein and arachidonic acid intake in the combined groups.



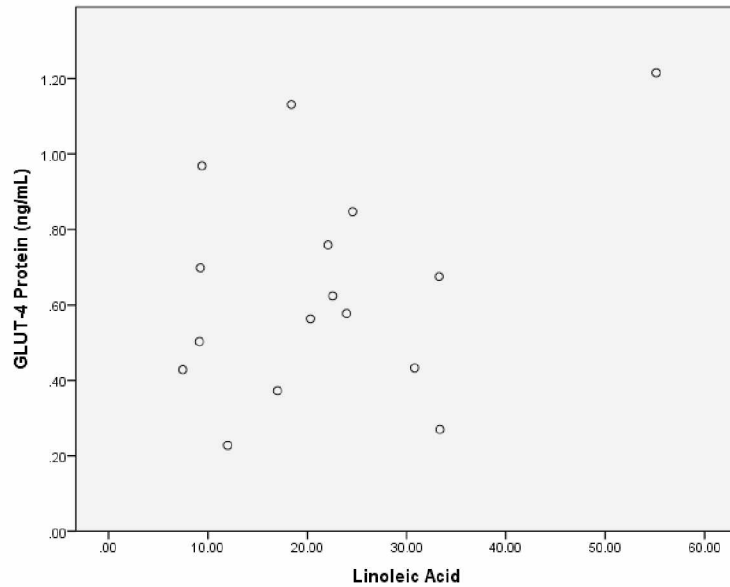
**FIGURE 2: Relationship Between GLUT-4 Protein and Saturated Fat Intake**

There is no association between GLUT-4 protein and saturated fat intake in the combined groups.



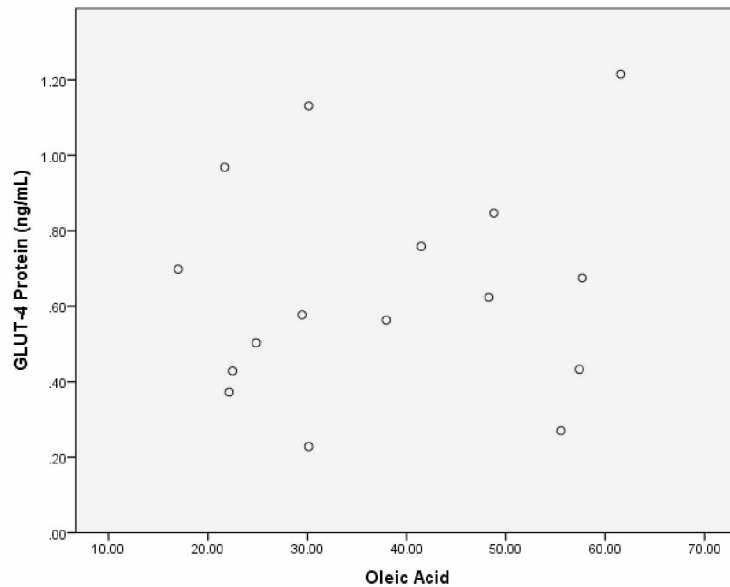
**FIGURE 3: Relationship Between GLUT-4 Protein and Palmitate**

There is no association between GLUT-4 protein and palmitate intake in the combined groups.



**FIGURE 4: Relationship Between GLUT-4 Protein and Linoleic Acid Intake**

There is no association between GLUT-4 protein and linoleic acid intake in the combined groups.



**FIGURE 5: Relationship Between GLUT-4 Protein and Oleic Acid Intake**

There is no association between GLUT-4 protein and oleic acid intake in the combined groups.

#### 4.2.2 Physical Activity Assessment

Despite a pre-screening of participants for physical activity levels, the International Physical Activity Questionnaire (IPAQ)-Short Form indicated that some of the participants in the sedentary group did in fact have moderate or high physical activity levels. A Mann-Whitney U test was conducted to determine if there were significant differences in physical activity, as assessed by the IPAQ-Short Form, between the two groups. MET-minutes per week were calculated which estimates the total energy cost of physical activity for a given week. Based on this calculation, participants were categorized as having low, moderate, or high activity levels. The distributions of MET-minutes per week and the categorical ranking of physical activity levels were different between groups, therefore median values could not be accurately compared, but mean ranks could be. The mean ranks between groups showed statistically significant differences for both methods of analyzing the IPAQ data (MET-minutes and categorical ranking

of physical activity). For MET-minutes per week,  $U=7.00$ ,  $p<0.0005$ ; for categorical physical activity levels,  $U=17.5$ ,  $p<0.0005$ , using an exact sampling distribution for each. These data confirm that the physical activity levels between groups were in fact highly significantly different which was intended in the study design.

#### 4.2.3 Biometric Assessment

Of all anthropometric measurements (Table 3), there was only a single significant difference between the groups for Body Mass Index ( $p=0.05$ ). All of the participants in the conditioned group had a BMI in a healthy range (18.6-25.0). In the sedentary group, ten participants had a healthy BMI, three were overweight (BMI = 25-29.9), and two were obese (BMI >30). There was no difference between groups for any other anthropometric measurements, however percent body fat and waist circumference trended toward significance ( $p = 0.10$  and  $0.06$ , respectively).

**TABLE 3: Body Mass and Composition**

Parameter	Conditioned	Sedentary	p-value
	Mean	Mean	
Body Mass Index ( $\text{kg/m}^2$ )*	21.7 (1.8)	24.8 (5.3)	0.05
Body Fat (%)	17.2 (8.0)	22.4 (8.9)	0.10
Waist Circumference (cm)	72.9 (5.8)	80.7 (13.9)	0.06
Basal Metabolic Rate (kcal/day)	1616 (212)	1718 (330)	0.31

Data are reported as means (standard deviations).

\*  $p \leq 0.05$ ; others non-significant at  $p > 0.05$

### 4.3 Laboratory Assessment

The laboratory assessment evaluated metabolic parameters as well as GLUT-4 protein and *SLC2A4* gene expression in PBMCs.

#### 4.3.1 Metabolic Parameters

There were no significant differences between the groups for glucose, HbA1c, insulin, or HOMA-IR (Table 4) although insulin and HOMA-IR approached significance ( $p = 0.14$  and  $0.15$ , respectively).

**TABLE 4: Glucose, Insulin, HbA1c, and HOMA-IR Comparison**

Parameter	Conditioned	Sedentary	p-value
	Mean	Mean	
Glucose (mg/dl)	90.8 (8.1)	93.4 (10.4)	0.43
HbA1c (% / mmol/mol)	5.1/32 (0.3)	5.0/31 (0.2)	0.49
	Mean Rank <sup>†</sup>	Mean Rank	
Fasting serum insulin ( $\mu$ IU/ml)	13.66	18.50	0.14
HOMA-IR <sup>†</sup>	13.69	18.47	0.15

Data are reported as means (standard deviations).

No significant differences between groups were identified for the glucose and insulin-related measures.

<sup>†</sup> Serum insulin and HOMA-IR had non-normal distributions therefore the Mann-Whitney U test was conducted. For both measures, the distributions between groups were not similar as assessed by visual

inspection therefore mean ranks were compared for analysis. For insulin  $U = 157.5$ ,  $z = 0.483$ ; for HOMA-IR  $U = 157.00$ ,  $z = 1.436$

<sup>†</sup> HOMA-IR calculation: [fasting serum glucose (mmol/L) \* fasting serum insulin (μIU/ml)] / 22.5

Total cholesterol, LDL, HDL, and triglycerides also were not different between groups (Table 5), but HDL and triglycerides approached significance ( $p = 0.10$  for both parameters). It is noteworthy that the Cholestech LDX which was used to measure lipid levels only detects triglycerides down to 45 mg/dl. LDL is calculated with the Friedwald formula using triglycerides. Six conditioned participants and two sedentary participants had undetectable levels of triglycerides with the methodology used. A value of 22.5 mg/dl was used (the midpoint between zero and the lowest detectable concentration) for those participants. This adds a level of error to both the triglyceride and LDL values so those data should be interpreted with caution.

**TABLE 5: Serum Lipids in Participant Groups**

Parameter	Conditioned	Sedentary	p-value
	Mean	Mean	
Total Cholesterol (mg/dl)	167.4 (25.3)	168.0 (24.2)	0.95
LDL (mg/dl) <sup>†</sup>	95.5 (32.8)	95.3 (16.9)	0.99
HDL (mg/dl)	68.0 (10.2)	59.5 (17.4)	0.10
	Mean Rank‡	Mean Rank	
Triglycerides (mg/dl) <sup>†</sup>	13.38	18.80	0.10

Data are reported as means (standard deviations).

No significant differences between groups were identified for lipids.

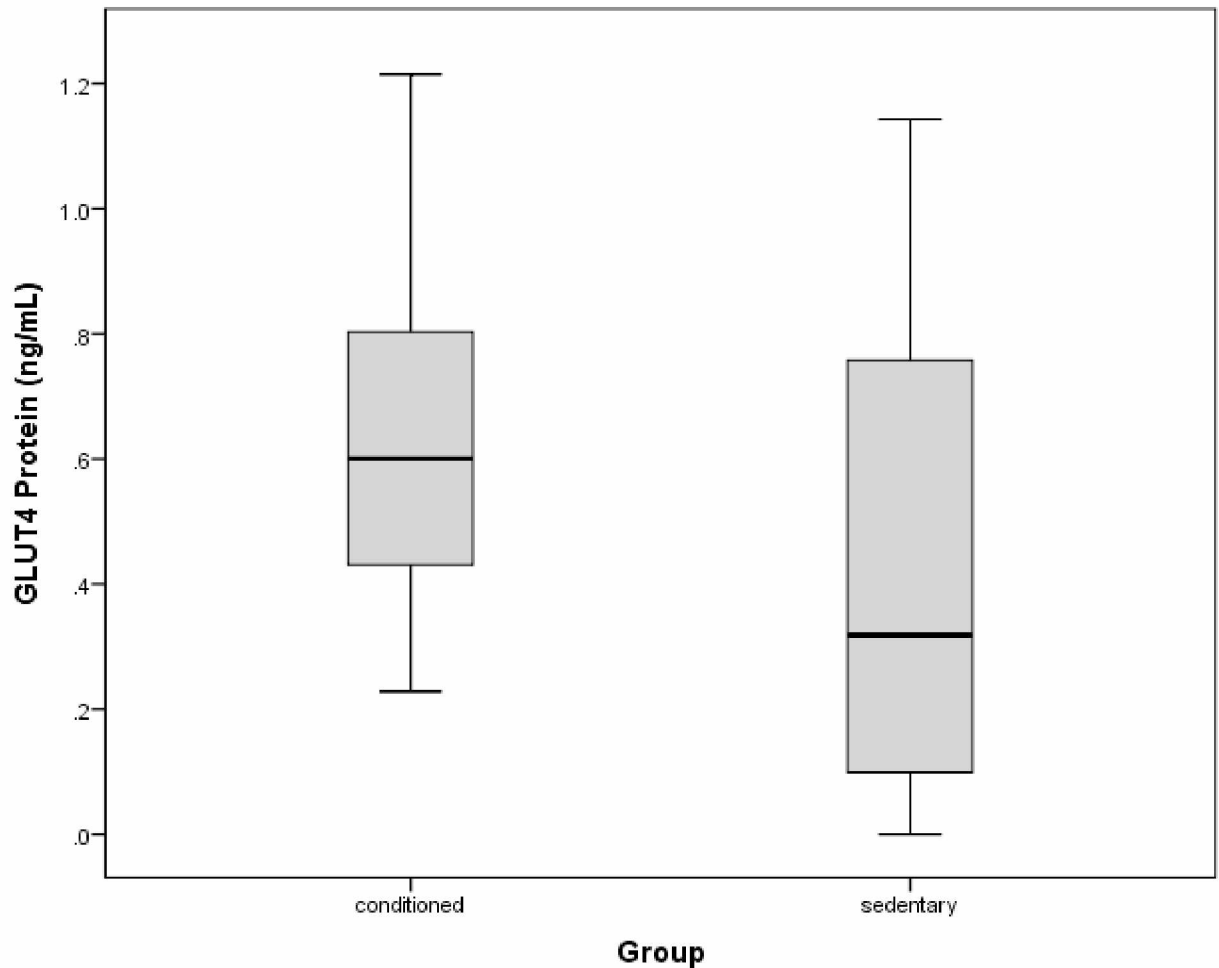


<sup>†</sup> 45 mg/dl is the lowest detectable level of triglycerides with the Cholestech LDX; LDL is calculated with the Friedewald formula using triglycerides. For participants with triglycerides lower than the detectable limit, a value of 22.5 was assigned and LDL was calculated using that value. This was done for six participants from the conditioned group and two participants from the sedentary group.

<sup>†</sup> Triglycerides had a non-normal distribution therefore the Mann-Whitney U test was conducted. The distributions between groups were not similar as assessed by visual inspection therefore mean ranks were compared for analysis.  $U = 162.000$  ,  $z = 1.675$

#### 4.3.2 GLUT-4 Protein

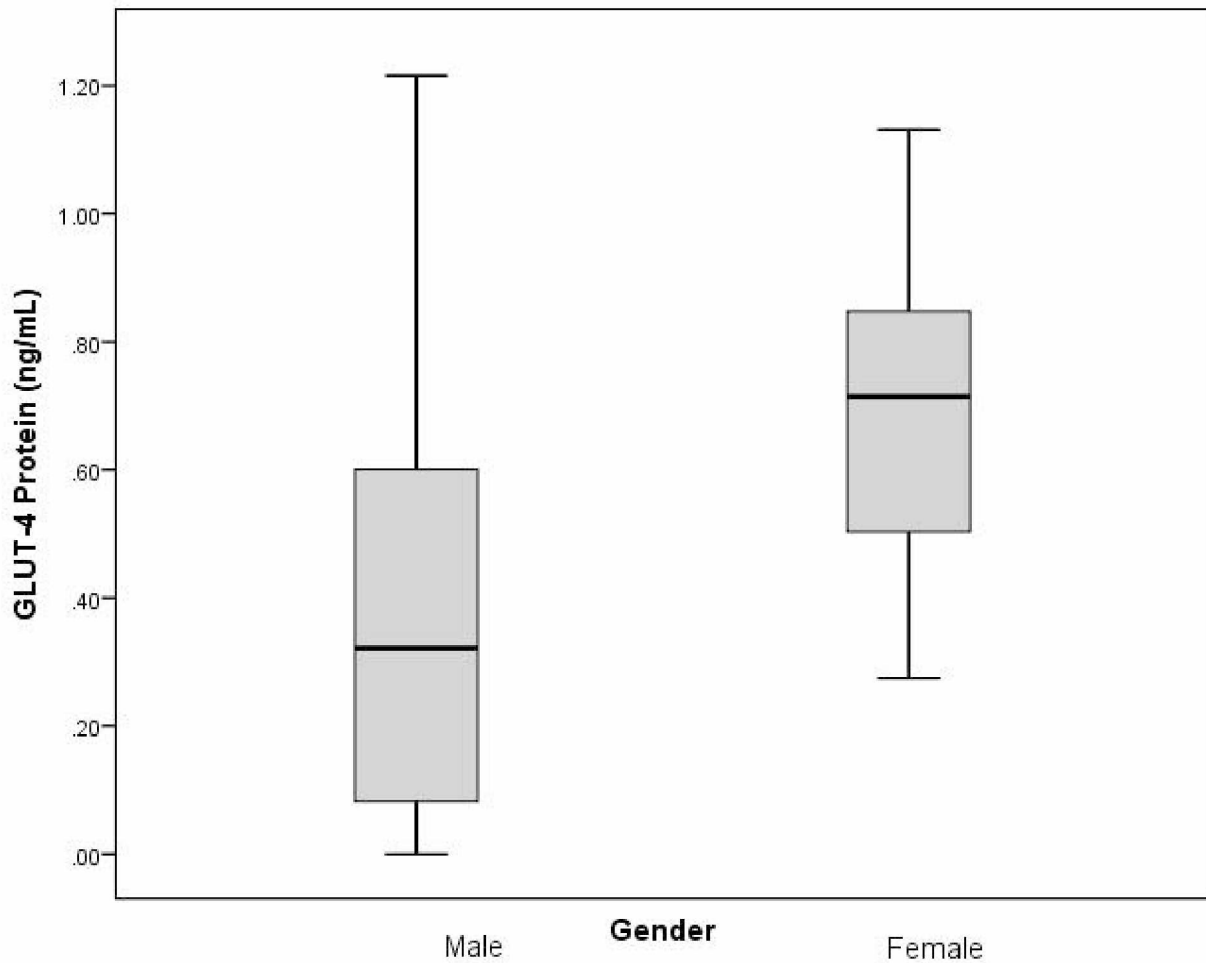
The conditioned group had a higher GLUT-4 on PBMC in fresh samples than the sedentary group; that difference was not statistically significant, but approached significance ( $p=0.07$ ) (Figure 6). The conditioned group ( $n=16$ ) had a mean of  $0.6433 \text{ ng/mL} \pm 0.2871$  compared to the sedentary group ( $n=13$  due to sample loss) mean of  $0.4057 \text{ ng/mL} \pm 0.3889$ .



**FIGURE 6: PBMC GLUT-4 Protein in Conditioned vs. Sedentary Participants**

GLUT-4 measured in ng/mL. The conditioned group had higher PBMC GLUT-4 on the cell surface than the sedentary group, although it was non-significantly different ( $p = 0.07$ ).

Females showed a higher mean GLUT-4 than males (0.7161 ng/mL vs. 0.3911 ng/mL) in the combined groups ( $p=0.01$ ) (Figure 7) and the sedentary group (0.6813 ng/mL vs. 0.2335 ng/mL;  $p=0.036$ ). In the conditioned group, GLUT-4 levels in females were higher than males (0.7379 ng/mL vs. 0.5488 ng/mL), although the difference was not significant ( $p=0.197$ ).



**FIGURE 7: PBMC GLUT-4 Protein in Males vs. Females**

GLUT-4 measured in ng/mL. Females from combined groups of participants had higher PBMC GLUT-4 on the cell surface than males ( $p = 0.01$ ).

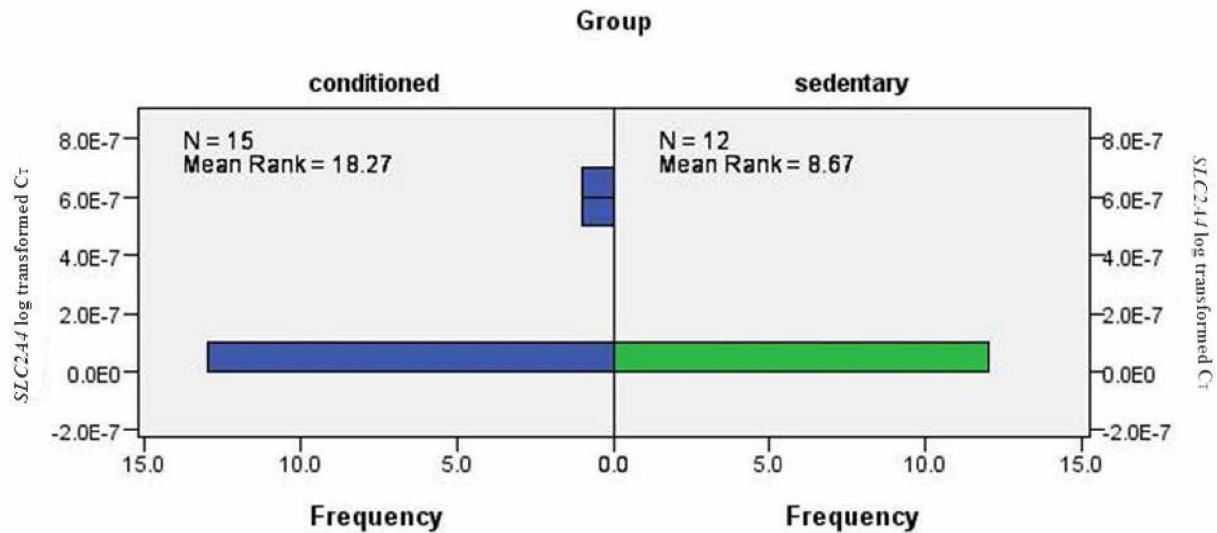
Pearson's correlation coefficient or Spearman's rank correlation coefficient were used to assess correlations between GLUT-4 and glucose, HbA1c, fasting insulin, HOMA-IR, BMI, and percent body fat. None of the parameters assessed showed a correlation with GLUT-4.

#### 4.3.3 Genomic Labs

*SLC2A4* mRNA was evaluated using real-time RT PCR. The  $C_T$  provides information on relative quantities of mRNA in the samples. *GAPDH* was utilized as an internal control.

The  $C_T$  for *GAPDH* was evaluated based on the methodology of Schmittgen and Livak to assess the quality of *GAPDH* as an internal control.<sup>111</sup> In this process, the  $C_T$  was log transformed to  $2^{-C_T}$ . The ratio of the  $2^{-C_T}$  between the conditioned and sedentary groups is 21.87 showing approximately a 22-fold higher expression in the conditioned group. This demonstrates that *GAPDH* is not a good internal control for evaluating effects of endurance exercise training. Unfortunately, there was insufficient sample to do an additional analysis with a different internal control gene. Subsequent analysis, therefore, uses the  $2^{-C_T}$  from the *SLC2A4* RT PCR without use of the *GAPDH*. Using this same methodology of calculating the ratio between the conditioned and sedentary group, the conditioned group showed a 15-fold higher expression of *SLC2A4* than the sedentary group.

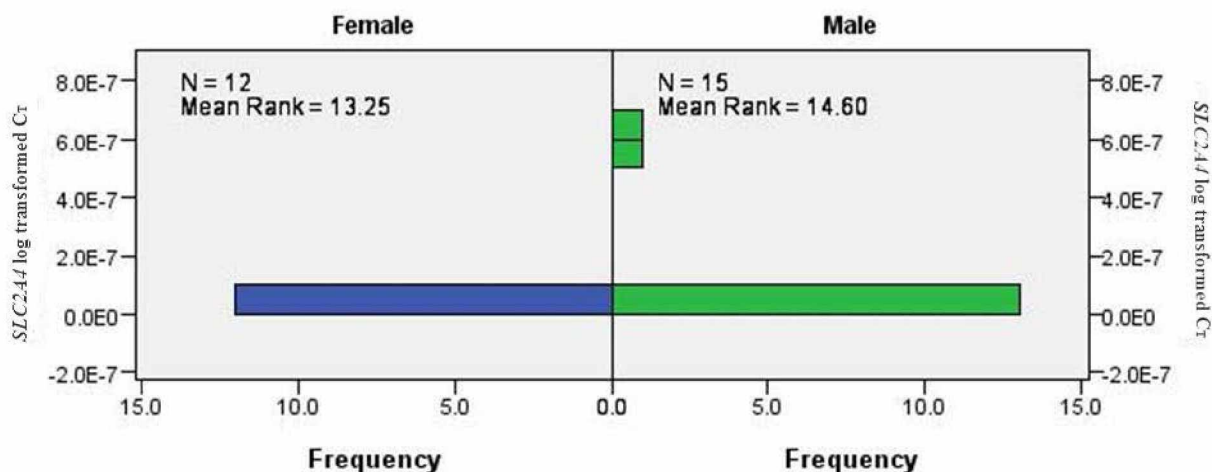
The relative quantities of *SLC2A4* mRNA, as evaluated by PCR, show that no groups have normal distribution of the  $2^{-C_T}$  values. Therefore the non-parametric Mann-Whitney U was used for analysis. By visual inspection of a histogram of the data, it does not appear that the data have the same shape therefore median values could not be compared, but mean ranks can. The mean rank of the conditioned group was 18.27 and the sedentary was 8.67 ( $p=0.001$ ). This indicates a difference in the distributions of the mean gene expression between the groups (Figure 8).



**FIGURE 8: *SLC2A4* mRNA Analysis for Conditioned vs. Sedentary Groups**

The distribution of mean ranks for the conditioned vs. sedentary groups is significantly different ( $p=0.001$ ).

Like the analysis between the conditioned and the sedentary groups, an analysis between sexes also shows a non-normal and non-similar distribution therefore mean ranks were compared. The mean rank was 13.25 for females and 14.60 for males showing no difference in distribution of mean gene expression between sexes (0.683) (Figure 9).



**FIGURE 9: *SLC2A4* mRNA Analysis for Females vs. Males**

The distribution of mean ranks is no different between sexes ( $p=0.683$ ).

*SLC2A4* mRNA was analyzed for an association between gene expression and dietary intake of specific nutrients and food groups including caloric intake, percent of calories from carbohydrate, fruit and vegetable servings, and intake of sugar, fiber, saturated fat, arachidonic acid, palmitate, oleic acid, and linoleic acid. None of these dietary variables show any type of relationship or association with *SLC2A4* mRNA.

SDS-PAGE with subsequent western blot was used to analyze GLUT-4 enhancer factor (GEF) between groups. Due to insufficient yield of protein in many samples, samples from only six of the conditioned group and three of the sedentary group were able to be used for analysis. A successful western blot showing clear GEF was not obtained.



## Chapter 5: Discussion and Conclusions

### 5.1 GLUT-4 Levels in Conditioned vs. Sedentary College Students

The present study compared GLUT-4 protein on PBMCs of conditioned and sedentary college students. These results were compared to two primary lines of literature: one which demonstrates that GLUT-4 is elevated in skeletal muscle tissue in response to exercise and a second which demonstrates PBMC GLUT-4 responds to insulin in a similar way that skeletal muscle responds to insulin. The goal was to determine whether to further evaluate PBMC as a proxy tissue for skeletal muscle for the study of GLUT-4.

The findings of this study demonstrate a non-significant, but noteworthy, difference in GLUT-4 on PBMC in conditioned compared to sedentary individuals. The conditioned athletes had higher GLUT-4 protein than their sedentary peers. Muscle biopsies were not obtained in this study so it cannot be concluded definitively that PBMC GLUT-4 quantity is a direct reflection of skeletal muscle GLUT-4 quantity, however the results do suggest this is a plausible hypothesis.

Samples for this study were obtained in the morning following a day of intense training for the conditioned group. Although a specific time frame following exercise was not built into the study design, it is known that the athletes reported for their blood draws approximately 15-19 hours following their last training session. The literature on the initiation of GLUT-4 elevation in skeletal muscle following exercise and the duration of maintenance of that elevation is inconclusive. Elevations may begin as early as three hours following exercise and may persist for as long as 36 hours. The time frame of blood draws for the current study are within that range, but it is difficult to predict whether analysis of PBMCs collected sooner after the training would have yielded higher or lower GLUT-4 levels.



Many studies on GLUT-4 changes in skeletal muscle in response to exercise do not account for variables such as body composition or dietary intake. In the current study, the sedentary group had a significantly higher BMI and a trend toward higher percent body fat and waist circumference than the sedentary group. The mean BMI within the sedentary group was however still within a “healthy weight” range (mean BMI 24.8). It cannot be ruled out that differences in body composition and weight had an impact on GLUT-4 quantity.

As would be expected from collegiate athletes, the calorie intake of the conditioned group was significantly higher than the sedentary group. Their fruit and vegetable and sugar intake was also significantly higher and the percent of calories from carbohydrates was higher although non-significantly ( $p=0.06$ ). Intake of calories, and particularly sugar and carbohydrates, stimulate insulin release. The study design did not provide a way to control for the possibility that increased GLUT-4 levels in the athletes were not somehow related to these dietary differences. The blood draws for PBMC analysis were conducted in a fasting state (minimum of 12 hours) so it would be expected that insulin stimulation from dietary intake would no longer be present. Nonetheless, the substantial difference in calorie and carbohydrate intake between the two groups is a limitation of the current study design yet one that could not be avoided when looking at groups with vastly different activity levels.

This study was limited by the fact that muscle biopsies were not obtained to directly compare muscle GLUT-4 to PBMC GLUT-4. Comparing the results to the existing literature however suggest that there may be a correlation between these two. Future studies with a design that allows for direct comparison are warranted. Using participants as their own control with pre- and post-exercise measures would help control for individual variabilities in factors such as caloric intake and body composition.

## 5.2 Correlation between PBMC GLUT-4 and Common Indicators of Insulin Resistance

Direct and indirect methods for assessing insulin sensitivity have been described.<sup>106</sup> The hyperinsulinemic euglycemic glucose clamp is a direct measure of insulin resistance. Using this procedure, after an overnight fast the individual would receive an insulin infusion at a constant rate. Simultaneously, a dextrose infusion would be given while sampling blood glucose levels every 5 to 10 minutes. The dextrose would be adjusted to keep blood glucose levels in a euglycemic range. A steady state must be achieved in this test which can take several hours. In these study conditions, the glucose infusion rate (GIR) should equal the glucose disposal rate since the test design is such that blood glucose levels are maintained. Clearly, this methodology is invasive and time consuming and therefore is not used in the clinical setting.

One of the most commonly used clinical methods for assessing insulin resistance is by assessing glucose tolerance. It is important to recognize that glucose intolerance and insulin resistance are not the same; however when assessing insulin resistance in a clinical setting the core question is typically whether the individual is able to maintain euglycemia regardless of glucose intake. An oral glucose tolerance would be used for this assessment.

Several surrogates of insulin resistance can be used clinically as well. One common method is to take fasting blood glucose and insulin levels. These values are then mathematically used to estimate insulin resistance. One limitation on any mathematical models using fasting insulin and glucose are that they only represent hepatic glucose and insulin utilization since they are taken in a basal state. In healthy individuals with normal glucose metabolism, this is going to be similar to peripheral insulin and glucose utilization, but in individuals with glucose metabolism disorders (such as diabetes or hypoglycemia) this may not be true. Another

limitation is that the lack of a standardized insulin assay prevents the development of standard cut-off values for diagnostic criteria.

The homeostasis model assessment for insulin resistance (HOMA-IR) is one commonly used surrogate based on a mathematical model. The calculation for HOMA-IR is  $\{[\text{fasting insulin } (\mu\text{U/ml}) \times [\text{fasting glucose (mmol/l)}]\} / 22.5$ . The 22.5 is a normalization factor which takes into account “normal” fasting glucose and “normal” fasting insulin levels. In a person with normal insulin sensitivity, the HOMA-IR would be about 1 due to normalization in the mathematical model with the 22.5. Fasting levels of insulin are not linear and therefore another methodology is to calculate  $\log(\text{HOMA-IR})$ . This may be more accurate in individuals with abnormal glucose tolerance or insulin sensitivity.

The present study used fasting glucose, insulin, HbA1c, and HOMA-IR to assess IR. A limitation of this study is that the direct measure of IR using a hyperinsulinemic euglycemic glucose clamp was not used. No difference between the two groups was found for any of the glucose or insulin parameters suggesting both groups had similar metabolic utilization of glucose. There was no correlation between PBMC GLUT-4 levels and any of the indicators of insulin resistance utilized in this study. This suggests that PBMC GLUT-4 may not be a good measure of insulin resistance in this population. It should be noted, however, that insulin and HOMA-IR data were non-normally distributed and a larger sample population may yield different results. There was no clinical indication of diabetes or pre-diabetes in any participant; individuals with impaired glucose tolerance may show different results. The findings of this study do not support PBMC GLUT-4 as a measure of insulin resistance however a modified study and participant sampling design may be warranted for further investigation.

### 5.3 Increased Transcription of *SLC2A4* in Conditioned vs. Sedentary College Students

The results of the present study, while not definitive, suggested a higher level of GLUT-4 protein in the conditioned participants compared to the sedentary. The next question of interest was whether genomic analysis would demonstrate that increased transcription of *SLC2A4* played a role in the mechanism of action.

While exploring this question, an unexpected finding, despite what existing literature might suggest, was that *GAPDH* does not appear to be a good internal control for assessing effects of exercise on gene expression. This became a limitation of data analysis. The most widely accepted methodology of analyzing real-time RT PCR is by using an internal control (“housekeeping gene”). Since the intended internal control, *GAPDH*, was significantly different between the groups, this methodology could not be used for analysis. Alternately, the log transformation of  $C_T$  was used ( $2^{-C_T}$ ). Although this is not the first choice of analysis, it does provide data on the raw mRNA levels. The input RNA volumes were standardized for cDNA synthesis which was then used for RT PCR. The conditioned group did indeed show higher expression of *SLC2A4* (15-fold) based on mRNA analysis when compared to the sedentary group.

A benefit of the finding related to *GAPDH* is that it identifies the need for additional methodology research in *SLC2A4* gene expression in response to exercise. Valid and reliable internal control genes for exercise-related research need to be identified for most accurate analysis of the data. Because physical activity has such widespread impact on physiological systems, this could prove challenging.

In addition to mRNA, this study attempted to assess MEF2 and HDAC5 binding to the *SLC2A4* promoter region. Again, the goal was to determine whether transcriptional activity in

PBMCs appeared to mimic what literature demonstrates occurs in skeletal muscle tissue. Chromatin was extracted using a commercial kit and then MEF2 and HDAC5 were immunoprecipitated using a commercial ChIP kit. There was insufficient yield at the end of this stage of the experiment to move forward with analyzing MEF2 and HDAC5 binding. Although this was an unfortunate outcome for the present study results, it does provide information about future methodology. Obtaining a blood cell count of samples prior to chromatin extraction would have allowed input into the experiment based on actual blood cell numbers as opposed to estimation of blood cell count based on blood volume. Additionally, a larger volume of blood collection would have allowed for more input material as well as an opportunity to replicate the experiment if needed. Based on this study, no conclusions can be drawn regarding whether MEF2 and HDAC5 activity on *SLC2A4* in PBMC correlate with activity in skeletal muscle tissue.

#### 5.4 Sex and GLUT-4 Protein

An unexpected, yet important, finding of this study was that females had higher GLUT-4 levels than males. Conditioning attenuated this effect to some extent. Many human studies on GLUT-4 upregulation in response to exercise have been conducted exclusively on male subjects. It may be that upregulation of GLUT-4 is more responsive to exercise in males than in females. There is little in the literature regarding the role of sex in GLUT-4 regulation in skeletal muscle. This needs to be explored both in skeletal muscle and PBMCs.

#### 5.5 Summary of Key Findings

In summary, several key findings from this study can guide future research:

- 1) GLUT-4 protein on PBMCs may respond to exercise in a similar fashion to myocyte GLUT-4. Muscle biopsy would be needed to validate the use of PBMCs as a proxy tissue for the study of skeletal muscle GLUT-4 response to exercise.
- 2) Diet should be accounted for in the study design of GLUT-4 regulation as it may play a role in transcription and/or translocation of GLUT-4.
- 3) *SLC2A4* transcription in PBMCs may occur in a similar fashion in response to exercise as *SLC2A4* transcription in skeletal muscle tissue. In order to accurately study this, an acceptable internal control gene needs to be identified.
- 4) Sex must be considered in studies of GLUT-4. Females demonstrate higher GLUT-4 levels and may have less GLUT-4 response to exercise than males.



## Chapter 6: Future Directions

Although the present study was unable to provide definitive conclusions regarding the use of PBMCs for the study of GLUT-4 regulation, the data do suggest this warrants further exploration. This chapter addresses study design considerations (Table 6) to investigate the use of PBMC as a proxy tissue for myocytes in the study of exercise and lifestyle impacts on GLUT-4.

### 6.1 Participant Selection

The literature is inconclusive as to whether GLUT-4 regulation in response to exercise differs between individuals with normal glucose metabolism and impaired glucose tolerance or diabetes. For this reason, it would be important to specify boundaries for blood glucose regulation in the participant selection criteria. Since the largest body of literature in humans addresses GLUT-4 regulation in individuals without impaired glucose tolerance that should be the selection criteria for an initial study. This would be consistent with the participants of the present study. If funding and time allowed, it would be insightful to have one group of participants with impaired glucose tolerance or type 2 diabetes and one group with normal glucose regulation.

Both chronic and acute exercise has an impact on GLUT-4 regulation in myocytes therefore participants with different pre-study exercise habits may introduce a confounding factor. It is proposed that participant selection criteria would exclude participants who engage in more than 20 minutes of moderate physical activity more than 3 times per week. It is



recommended to use the IPAQ short form as a pre-screening tool to verify that participants are similar in their exercise habits.

Medical conditions such as thyroid disorder and pregnancy would be exclusion criteria due to the potential for affecting GLUT-4 regulation. Due to the key role the liver plays in glucose metabolism, individuals with known liver disease or dysfunction would also be excluded. Individuals taking corticosteroids or hypoglycemic medications would be excluded. All prospective participants would need to be medically able to participate in the exercise portion of the intervention.

Based on the results of the present study that sex likely has an impact on GLUT-4 levels and potentially on GLUT-4 regulation, sex must be accounted for. Ideally, one-half of the participants would be male and one-half female. Dividing into these subgroups however reduces the statistical power therefore if a large enough sample size is not feasible it is recommended to select only one sex. Since the proposed study would be early work in this area, it is recommended that if only one sex can be used that it be males since the majority of literature currently includes exclusively males. Subsequent studies could look to replicate the results in females.

## 6.2 Study Protocol

The study would be designed to compare PBMC GLUT-4 to myocyte GLUT-4 in an attempt to validate PBMC as a proxy tissue for myocytes for the study of GLUT-4. An exercise intervention would be provided and laboratory data collected and analyzed to assess effects of a single exercise bout (30 and 60 minutes) and two weeks of moderate exercise.

Previous studies have utilized exercise at a  $\text{VO}_2$  max ranging from 60-75% with 75% being the most common.<sup>21,24,28,78,112</sup> For the proposed study, an exercise intensity of 75%  $\text{VO}_2$  max is suggested. The exercise could be done on a treadmill or exercise bicycle.

Muscle biopsy would be from vastus lateralis muscle as described in previous studies.<sup>21-24,28,77,78,112</sup> Collection of PBMCs would be done with the same protocol as the present study however three 8-ml tubes would be collected instead of one to ensure adequate sample for all procedures and potential need for replicates.

Dietary intake could affect GLUT-4 levels, particularly if caloric intake or carbohydrate intake is high. Ideally, participants would be given food boxes for the duration of the study that provided them a modest carbohydrate diet (50-60% carbohydrate) meeting their individual calorie needs as determined by indirect calorimetry. If it was not possible to have that level of control, participants could be educated on a modest carbohydrate diet so they were not limiting carbohydrates nor eating excessive amounts. Participants could be asked to record their food intake in the ASA24 self-administered 24-hour recall system daily to assess total caloric intake and macronutrient distribution.

**TABLE 6: Overview of Proposed Future Study Protocol**

Time Frame	Interventions/ Protocols	Lab Tests / Procedure
Prior to initiation of study (1-3 weeks prior)	<ul style="list-style-type: none"> <li>None</li> </ul>	<ul style="list-style-type: none"> <li>Pre-screening questionnaires (medical, exercise)</li> <li>Fasting blood glucose and HbA1c to verify normal</li> </ul>

**TABLE 6 Cont.**

		<p>blood glucose levels</p> <ul style="list-style-type: none"> <li>• VO<sub>2</sub> max</li> <li>• Resting Metabolic Rate via indirect calorimetry (Parvo Metabolic Cart)</li> <li>• Height, weight, and body composition via BodPod</li> <li>• Participant orientation and training on diet recording tool</li> </ul>
Day 1	<ul style="list-style-type: none"> <li>• 30 minutes exercise</li> <li>• Exercise done at least 4 hours after eating to minimize any effect of food on GLUT-4</li> <li>• Sample schedule: participant eats breakfast at 7 AM, comes to clinic at 11 AM for blood draw and muscle biopsy, performs 30 minute cycling, returns to clinic at 6 PM for second blood</li> </ul>	<ul style="list-style-type: none"> <li>• Muscle biopsy</li> <li>• PBMC</li> <li>• Collection of each done pre-exercise, 6 hours post exercise, and 18 hours post-exercise</li> </ul>

**TABLE 6 Cont.**

	draw and muscle biopsy, then returns to clinic at noon on following day for third blood draw and muscle biopsy.	
Days 2-4	<ul style="list-style-type: none"> <li>• 30 minutes exercise daily</li> </ul>	<ul style="list-style-type: none"> <li>• None</li> </ul>
Day 5	<ul style="list-style-type: none"> <li>• Rest</li> </ul>	<ul style="list-style-type: none"> <li>• None</li> </ul>
Day 6	<ul style="list-style-type: none"> <li>• 60 minutes exercise</li> <li>• Exercise done at least 4 hours after eating to minimize any effect of food on GLUT-4</li> <li>• Sample schedule: participant eats breakfast at 7 AM, comes to clinic at 11 AM for blood draw and muscle biopsy, performs 60 minute cycling, returns to clinic at 6:30 PM for second blood draw and muscle biopsy, draw and muscle biopsy,</li> </ul>	<ul style="list-style-type: none"> <li>• Muscle biopsy</li> <li>• PBMC</li> <li>• Collection of each done pre-exercise, 6 hours post exercise, and 18 hours post exercise</li> </ul>

**TABLE 6 Cont.**

	then returns to clinic at 12:30 on following day for third blood draw and muscle biopsy.	
Days 7-9	<ul style="list-style-type: none"> <li>• 60 minutes exercise daily</li> </ul>	<ul style="list-style-type: none"> <li>• none</li> </ul>
Day 10	<ul style="list-style-type: none"> <li>• Rest</li> </ul>	<ul style="list-style-type: none"> <li>• none</li> </ul>
Day 11-14	<ul style="list-style-type: none"> <li>• 60 minutes exercise daily</li> </ul>	<ul style="list-style-type: none"> <li>• none</li> </ul>
Day 15	<ul style="list-style-type: none"> <li>• none</li> </ul>	<ul style="list-style-type: none"> <li>• Muscle biopsy</li> <li>• PBMC</li> <li>• Collection of each done at 24 hours following last exercise session</li> <li>• VO<sub>2</sub> max</li> </ul>

### 6.3 Laboratory Analysis

Laboratory analysis would focus on GLUT-4 protein and *SLC2A4* mRNA content in myocytes and PBMCs. The data would be analyzed from two primary perspectives:

- 1) To determine whether the quantity of GLUT-4 protein and *SLC2A4* mRNA in myocytes correlates with the quantity in PBMC in a given individual at a given point in time.

- 2) To elucidate whether the effects of exercise impact GLUT-4 protein and *SLC2A4* mRNA in a similar fashion in myocytes and PBMCs.

#### 6.3.1 GLUT-4 Protein

The PBMCs and myocytes would be analyzed via flow cytometry to obtain a cell count and cell surface GLUT-4 protein. Obtaining a cell count would allow expression of GLUT-4 per a particular quantity of cells. The present study used ELISA for analyzing GLUT-4 however the vast majority of literature has utilized flow cytometry. ELISA is attractive due to the ease of the procedure and ability to store cells for a longer period of time prior to analysis. For this reason, it is suggested that ELISA be conducted additionally so the results from flow cytometry and ELISA could be compared to help validate ELISA as a methodology for assessing cell surface GLUT-4 protein.

#### 6.3.2 *SLC2A4* mRNA

*SLC2A4* mRNA would be analyzed with real time RT-PCR as was done in the methods of the present study however an internal control gene would be validated prior to running the experimental protocol. Candidate internal controls include cyclophilin, beta-actin, and 28S rRNA because of their use in similar human studies.<sup>24,28,77,112</sup> An internal control would be selected and utilized following this validation.

#### 6.4 Conclusions

GLUT-4 regulation is a key aspect of blood glucose control. A better understanding of how environmental and pharmacological treatments may be able to upregulate GLUT-4 would

be valuable. In order for human research to progress more rapidly in that area, it would be beneficial to have an easily obtained cell type to study. The present study suggests that PBMCs may be a viable cell type for this purpose. The proposed study would allow further exploration of this hypothesis.

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Appendix A  
Institutional Review Board Approval

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**Institutional Review Board**

909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

September 30, 2013

To: Kriya Dunlap, PhD

Principal Investigator

From: University of Alaska Fairbanks IRB

Re: [492213-4] A pilot study: GLUT-4 and conditioning

Thank you for submitting the Amendment/Modification referenced below. The submission was handled by Administrative Review under the requirements of 45 CFR 46.110, which identifies the categories of

research eligible for expedited review.

Title: A pilot study: GLUT-4 and conditioning

Received: September 30, 2013

Expedited Category: 2 *and* 7

Action: APPROVED

Effective Date: September 30, 2013

Expiration Date: August 7, 2014

Required Information:

Minor modification was made and administratively approved as requested by reviewer in package 3 (previous package).



This action is included on the October 2, 2013 IRB Agenda.

*No changes may be made to this project without the prior review and approval of the IRB. This includes, but is not limited to, changes in research scope, research tools, consent documents, personnel, or record storage location.*

- 1 - Generated on IRBNet

Appendix B  
Demographic and Medical Questionnaire

**Demographic and Medical Screening Questionnaire**

**Date:** \_\_\_\_\_ **Study #** \_\_\_\_\_

**Introduction to general screening questionnaire:** We would like to ask you a few general screening questions and some specific questions about your medical history and medications you're currently taking. This will take about 10 minutes. Please leave a blank for any question you do not wish to answer or do not know the answer.

**Please Circle One.**

01. Sex:

- 1. M
- 2. F

02. Where do you currently live? On-campus housing\_\_\_\_, Off-campus\_\_\_\_, with your parents\_\_\_\_?

03. Who currently lives in your household and how are they related to you? (Circle **ALL** that apply)

Relationship	How Many?	Relationship	How Many?	Relationship	How Many?
1. Wife		8. Sister		15. Niece	
2. Husband		9. Grandmother		16. Nephew	
3. Daughter		10. Grandfather		17. Cousin	
4. Son		11. Granddaughter		18. Girlfriend	
5. Mother		12. Grandson		19. Boyfriend	
6. Father		13. Aunt		99. Other	
7. Brother		14. Uncle			

04. What is your academic standing (freshman, sophomore etc.)? \_\_\_\_\_

05. Where do you most often eat? (Please circle only one)

- 1. Cook for myself at home
- 2. Eat at campus dining facility
- 3. Eat out in town
- 4. Eat with my family at home

06. Do you receive assistance to pay for food (i.e., food stamps or WIC coupons)?

1. Yes
2. No
3. No Response

07. What is your race? (Circle all that apply)

1. Alaska Native
2. White
3. Black or African American
4. Asian
5. Other \_\_\_\_\_

08. How often do you eat Alaskan wild fish, seafood, game or berries?

1. A lot
2. Some
3. Not at all
4. No Response

09. Have you **ever used** any type of tobacco products?

1. Yes
0. No (Skip to question 17)

010. I have **used** tobacco products

1. But no longer use (Go to question 011)
2. Have quit some tobacco products but still use tobacco (Go to question 011)
3. And currently use tobacco (Go to question 014)

Please give answers for each type of tobacco product you **used but quit**: (Put a "0" in the **first** column if you never used that type of tobacco)

	(i)	(ii)
(iii)	Avg. <u>number</u> used/day	Age when <u>started</u> use
Age when <u>quit</u>		
Type of tobacco used		
011. Smoked cigarettes	# cigs _____	_____
012. Chewed commercially-prepared (i.e. Levi-Garrett or Redman)	# chews _____	_____
013. Used Snuff (i.e. Copenhagen or Skoal)	# of dips _____	_____

(If no longer use tobacco, go to question 35)

Please give answers for each type of tobacco you **now use**: (Put a "0" in the **first** column if you never used that type of tobacco)

		(i) Avg. <u>number</u> use/day	(ii) Age when <u>started</u> use
014. Smoke cigarettes	#cigs	_____	_____
015. Chew commercially-prepared (i.e. Levi-Garrett or Redman)	# chews	_____	_____
016. Use snuff (i.e. Copenhagen or Skoal)	# of dips	_____	_____
017. Do you consume alcohol?			
1. Yes	How many drinks per week	_____	
2. No			

### The following questions are about your medical history.

018. Are you currently taking any medications prescribed by a health care provider?

1. Yes Which ones? \_\_\_\_\_

0. No

019. Are you taking vitamins or other supplements?

1. Yes Which ones? \_\_\_\_\_

0. No

020. Have you or a family member **related by blood** (parents [p], siblings [s], children [C]) ever been diagnosed with any of the following (DO NOT provide names of family members, only the **number** of family members):

Self	Yes	No	Unknown	Family member(s)	Yes	No	Unknown	How many of each?
	1	0	8888		1	0	8888	P / S / C
Arthritis	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Arthritis	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Osteo	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Osteo	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Rheumatoid	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Rheumatoid	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Asthma	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Asthma	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Cancer		<input type="checkbox"/>	<input type="checkbox"/>	Cancer		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Breast	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Breast	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Colon	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Colon	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Lung	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Lung	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Ovarian		<input type="checkbox"/>	<input type="checkbox"/>	Ovarian		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Prostate		<input type="checkbox"/>	<input type="checkbox"/>	Prostate		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Stomach		<input type="checkbox"/>	<input type="checkbox"/>	Stomach		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Other _____	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Other _____	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Diabetes Type I	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Diabetes Type I	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Diabetes Type II	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Diabetes Type II	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Gestational Diabetes		<input type="checkbox"/>	<input type="checkbox"/>	Gestational Diabetes		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Depression		<input type="checkbox"/>	<input type="checkbox"/>	Depression		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Gall Bladder/Stones	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Gall Bladder/Stones	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Heart Attack		<input type="checkbox"/>	<input type="checkbox"/>	Heart Attack		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
High Cholesterol	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	High Cholesterol	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Hypertension		<input type="checkbox"/>	<input type="checkbox"/>	Hypertension		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Kidney failure		<input type="checkbox"/>	<input type="checkbox"/>	Kidney failure		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Osteoporosis		<input type="checkbox"/>	<input type="checkbox"/>	Osteoporosis		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Overweight		<input type="checkbox"/>	<input type="checkbox"/>	Overweight		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Stroke		<input type="checkbox"/>	<input type="checkbox"/>	Stroke		<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Thyroid Disorder	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	Thyroid Disorder	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	____/____/____
Others _____				Others _____				____/____/____
Pacemaker	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>					

**DO NOT MEASURE BODY FAT ON TANITA IF "YES" TO PACEMAKER**

**Now we'd like to collect some information on your body measurements.**

021. Height: \_\_\_\_\_ in. (measured by researcher)

**Tanita Data:**

**Results from Impedance Analyzer**

022. Weight: \_\_\_\_\_ lbs.

*(Please tape tanita printout*

*here)*

023. BMI: \_\_\_\_\_



024. Percent body fat: \_\_\_\_\_ %

025. BMR: \_\_\_\_\_ Kcal

026. Impedance: \_\_\_\_\_

027. Fat Mass: \_\_\_\_\_ lb.

028. FFM: \_\_\_\_\_ lb.

029. TBW: \_\_\_\_\_ lb.

030. Height entered into Tanita: \_\_\_\_\_ ft. \_\_\_\_\_ in.

031. Waist circumference: \_\_\_\_\_ cm    2<sup>nd</sup> Measure: \_\_\_\_\_    3<sup>rd</sup> Measure: \_\_\_\_\_

032. Blood pressure: 1<sup>st</sup> measurement: Systolic \_\_\_\_\_ Diastolic \_\_\_\_\_ Pulse \_\_\_\_\_

2<sup>nd</sup> measurement: Systolic \_\_\_\_\_ Diastolic \_\_\_\_\_ Pulse \_\_\_\_\_

3<sup>rd</sup> measurement: Systolic \_\_\_\_\_ Diastolic \_\_\_\_\_ Pulse \_\_\_\_\_

Average measurement: Systolic \_\_\_\_\_ Diastolic \_\_\_\_\_ Pulse \_\_\_\_\_

### Data from Cholestech:

#### Analysis

033. Triglycerides: \_\_\_\_\_ mg/dl  
*here)*

034. Total Cholesterol: \_\_\_\_\_ mg/dl

035. Glucose: \_\_\_\_\_ mg/dl

036. HDL: \_\_\_\_\_ mg/dl

037. LDL: \_\_\_\_\_ mg/dl

038. VLDL: \_\_\_\_\_ mg/dl

039. HbA1c: \_\_\_\_\_ %

040. Cholestech Date: \_\_\_\_\_

041. Cholestech Time: \_\_\_\_\_

042. NPO after (last eat) Date: \_\_\_\_\_

043. NPO after (last eat) Time: \_\_\_\_\_

#### Results from Blood

*(Please place Cholestech printout*



#### **Fasting Status**

- ☐ Fasting total (at least 8 hrs)
- ☐ Fasting except black coffee (no cream or sugar)
- ☐ Non-fasting

044. Blood drawn at Date: \_\_\_\_\_

045. Blood drawn at Time: \_\_\_\_\_



## Appendix C

### International Physical Activity Questionnaire Short Form (IPAQ-SQ)

Study # \_\_\_\_\_

#### INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in effects of physical activity on insulin signaling. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

\_\_\_\_\_ **days per week**

☐

No vigorous physical activities → **Skip to question 3**

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

\_\_\_\_\_ **hours per day**

\_\_\_\_\_ **minutes per day**

☐

Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

\_\_\_\_\_ **days per week**

☐ No moderate physical activities → ***Skip to question 5***

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

\_\_\_\_\_ **hours per day**

\_\_\_\_\_ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

\_\_\_\_\_ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

\_\_\_\_\_ **hours per day**

\_\_\_\_\_ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

\_\_\_\_\_ **hours per day**

\_\_\_\_\_ **minutes per day**

☐

Don't know/Not sure

**This is the end of the questionnaire, thank you for participating.**

## Appendix D

### Western Blot Optimization Trials

#### Western Blotting Antibody Concentrations / Reaction Optimization

Anti-GEF / SLC2A4RG primary antibody; anti-rabbit secondary antibody

Experiment	Primary	Secondary	Notes / Results on Imaging
1	1:1000	1:2000	Very faint, if any, band was seen where would be expected for GEF at about 41 kDa.
2	1:500	1:2000	No real improvement over experiment 1.
4	1:1000	1:2000	Very faint lines observed at about 41 kDa, but a significant amount of non-specific binding still observed on the blot.
5	1:1000	1:100,000	No visible lines seen where GEF expected to be
6	1:1000	1:10,000	No clear lines; continues to be significant background noise in the image.

Anti-GLUT4 primary antibody; anti-mouse secondary antibody

Experiment	Primary	Secondary	Notes
1	1:500	1:2000	Bands were seen where expected for GLUT4 at about 54 kDa, however there

			were large amounts of other non-specific binding / noise.
3	1:500	1:4000	Continued to have large amounts of non-specific binding/noise.

On experiment 1, both primary and secondary antibodies were added simultaneously. The membrane was then stripped and all further experiments (2-6) were conducted with one primary and one secondary antibody at a time.

Experiment 1 had large amounts of non-specific binding/noise and little, if any, visible band for GEF. It was suspected that there was too low of a concentration of primary antibody for GEF and the mouse secondary antibody was too concentrated.

The membrane was stripped between each subsequent experiment.

For experiment 2, the anti-GEF antibody concentration was increased and the secondary was left at 1:2000. Very faint lines were observed where expected, but there continued to be large amounts of non-specific binding/noise in the image.

For experiment 3, the anti-GLUT4 antibody was left at a concentration of 1:500, but the concentration of the secondary was reduced to 1:4000. This did not appear to improve the non-specific binding/noise in the image.

For experiments 4-6, the anti-GEF antibody was used, with a concentration of 1:1000. The secondary antibody was adjusted in concentration as shown above.